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(71) Applicant (for all designated States except US): **FIBRO-  
GEN, INC.** [US/US]; 225 Gateway Boulevard, South San  
Francisco, CA 94080 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **CHANG, Robert**,  
C. [US/US]; 2155 Edgecourt Drive, Burlingame, CA  
94010 (US). **KIVIRIKKO, Kari, I.** [FI/FI]; Parkkisen-  
ranta 5, FIN-90650 Oulu (FI). **NEFF, Thomas, B.**  
[US/US]; 190 Glenwood Avenue, Atherton, CA 94027  
(US). **OLSEN, David, R.** [US/US]; 276 Hledge Road,  
Menlo Park, CA 94025 (US). **POLAREK, James, W.**  
[US/US]; #3, A Dock, Waldo Point Harbor, Sausalito, CA  
94965 (US).

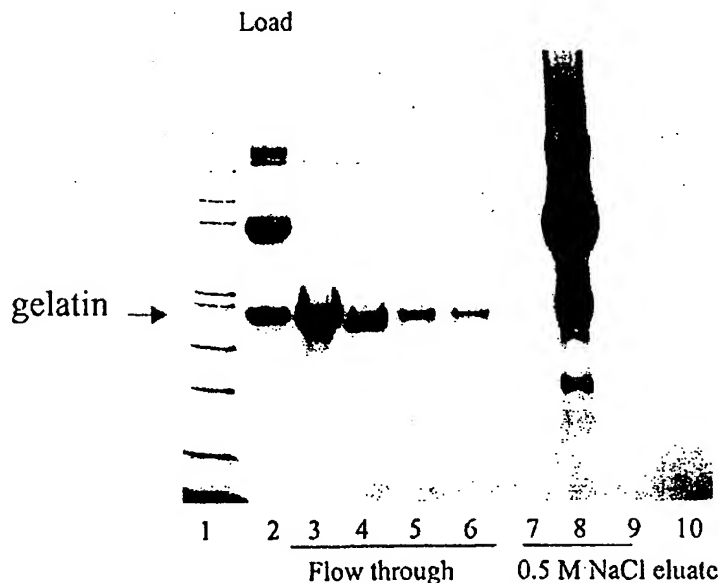
(74) Agents: **PRICE, Leanne, C.**; Fibrogen, Inc., 225 Gateway  
Boulevard, South San Francisco, CA 94080 et al. (US).

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(54) Title: **RECOMBINANT GELATIN IN VACCINES**



(57) Abstract: The present invention relates to vaccines comprising recombinant gelatin, to methods of producing and using such vaccines, and to vaccination kits.

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**RECOMBINANT GELATIN IN VACCINES**

This application claims the benefit of U.S. Provisional Application Nos. 60/204,437, filed 15 May 2000, and 60/165,114, filed 12 November 1999, the specifications of which are incorporated herein by reference in their entireties.

10

**FIELD OF THE INVENTION**

This invention relates to vaccines, and, specifically, to vaccine formulations comprising recombinant gelatin.

15

**BACKGROUND OF THE INVENTION**

Vaccines are preparations of killed or modified microorganisms, living attenuated organisms, or living fully virulent organisms, or any other infective agent, including, but not limited to peptides, proteins, biological macromolecules, or nucleic acids, natural, synthetic, or semi-synthetic, capable of stimulating an immune response when administered to a subject.

20 Vaccines are provided in order to prevent or temper the severity of subsequent exposure to or infection with a similar microorganism. In live attenuated vaccines, the microorganism has typically been treated to produce an avirulent strain capable of inducing protective immunity. In inactivated vaccines, the infectious microbial nucleic acid components are destroyed prior to administration, without affecting the antigenicity or immunogenicity of the viral coat or  
25 bacterial outer membrane proteins of the microorganism.

Vaccination has accomplished radical changes in global health. For example, in 1980, the World Health Organization declared smallpox eradicated as a result of international vaccination efforts. Diphtheria, whooping cough, and measles, responsible for high rates of  
30 childhood mortality, are the focus of established infant and childhood immunization programs in industrialized countries, and there are continuing efforts to establish such programs in developing countries. Immunizations protect children against diseases including polio, measles, mumps, rubella (German measles), diphtheria, pertussis (whooping cough), chicken pox, hepatitis B, and *Haemophilus influenzae* type b. Current immunization schedules can  
35 start within 12 hours of birth, and require multiple immunizations during the first two years. Administration of vaccines can occur at various points throughout a person's lifetime, for example, seasonal vaccines, e.g., "flu vaccine," and vaccines administered to travelers, etc.

5 Vaccines can be administered in a number of ways. Currently, vaccines are primarily delivered through injection or oral administration. Vaccine formulations can include, for example, adjuvants to enhance the immune response, so that less vaccine is needed to produce the desired protective immune response. Various excipients and carriers, contributing to the consistency and deliverability of the vaccine, can also be provided.

10

Vaccines can include various components included within the vaccine formulation to maintain the stability of the vaccine. Such stabilizers are intended to maintain the integrity of the vaccine, preserving the vaccine's ability to elicit a protective immune response upon administration. Stabilizers used in vaccine formulations include, but are not limited to, 15 chemical and biological agents that perform a variety of functions, such as, for example, detergents, buffers, salts, sugars, and various protein components, including gelatin, specifically, hydrolyzed gelatin.

#### Manufacture of Gelatin

20 Gelatin is a derivative of collagen, a principal structural and connective protein in animals. Gelatin is derived from denaturation of collagen and contains polypeptide sequences having Gly-X-Y repeats, where X and Y are most often proline and hydroxyproline residues. These sequences contribute to triple helical structure and affect the gelling ability of gelatin polypeptides. Currently available gelatin is extracted through processing of animal hides and 25 bones, typically from bovine and porcine sources. The biophysical properties of gelatin make it a versatile material, widely used in a variety of applications and industries. Gelatin is used, for example, in numerous pharmaceutical and medical, photographic, industrial, cosmetic, and food and beverage products and processes of manufacture. Gelatin is thus a commercially valuable and versatile product.

30

Gelatin is typically manufactured from naturally occurring collagen in bovine and porcine sources, in particular, from hides and bones. In some instances, gelatin can be extracted from, for example, piscine, chicken, or equine sources. Raw materials of typical gelatin production, such as bovine hides and bones, originate from animals subject to government-certified 35 inspection and passed fit for human consumption. There is concern over the infectivity of this raw material, due to the presence of contaminating agents such as transmissible spongiform encephalopathies (TSEs), particularly bovine spongiform encephalopathy (BSE), and scrapie, etc. (See, e.g., Rohwer, R.G. (1996), Dev Biol Stand 88:247-256.) Such issues are especially critical to gelatin used in pharmaceutical and medical applications.

5

Recently, concern about the safety of these materials, a significant portion of which are derived from bovine sources, has increased, causing various gelatin-containing products to become the focus of several regulatory measures to reduce the potential risk of transmission of bovine spongiform encephalopathy (BSE), linked to new variant Creutzfeldt-Jakob disease (nvCJD), a fatal neurological disease in humans. There is concern that purification steps currently used in the processing of extracting gelatin from animal tissues and bones may not be sufficient to remove the likelihood of infectivity due to contaminating SE-carrying tissue (i.e., brain tissue, etc.). U.S. and European manufacturers specify that raw material for gelatin to be included in animal or human food products or in pharmaceutical, medical, or cosmetic applications must not be obtained from a growing number of BSE countries. In addition, regulations specify that certain materials e.g., bovine brain tissue, are not used in the production of gelatin.

Current production processes involve several purification and cleansing steps, and can require harsh and lengthy modes of extraction. The animal hides and bones are treated in a rendering process, and the extracted material is subjected to various chemical treatments, including prolonged exposure to highly acidic or alkaline solutions. Numerous purification steps can involve washing and filtration and various heat treatments. Acid demineralization and lime treatments are used to remove impurities such as non-collagenous proteins. Bones must be degreased. Additional washing and filtration steps, ion exchanges, and other chemical and sterilizing treatments are added to the process to further purify the material. Furthermore, contaminants and impurities can still remain after processing, and the resultant gelatin product must thus typically be clarified, purified, and often further concentrated before being ready for use.

30

Commercial gelatin is generally classified as type A or type B. These classifications reflect the pre-treatment extraction sources receive as part of the extraction process: Type A is generally derived from acid-processed materials, usually porcine hides, and type B is generally derived from alkaline- or lime-processed materials, usually bovine bones (ossein) and hides.

35

In extracting type A gelatin, the process generally involves subjecting fresh or frozen porcine hides to successive washings with water and treatments with dilute acids. The acid-treated skins are washed again and are then subject to repeated extraction steps in which they are

- 5 treated with hot water, partially hydrolyzing the collagen present. The resultant extracts, dilute solutions of gelatin, are filtered and evaporated, and the resultant concentrates are allowed to cool or chilled to a gel. The gel is subsequently treated in drying tunnels, or by continuous dryers or other drying devices.
- 10 In the limed process, type B gelatin is derived from donor hides and skin trimmings washed and then treated with lime. The lime treatment can take as long as from one to three months, and is usually around sixty days. The limed hides are washed and treated with dilute acids. The hides are then hydrolyzed with hot water and the resulting extracts are processed as described above for the acid-treatment process.
- 15 Type B gelatin can also be processed from ossein sources. The hard bones are washed, degreased, and leached with successive treatments of dilute acids, such as hydrochloric acid. The acid treatment reacts with the mineral contents of bone, which are removed along with the acidic solution, leaving ossein, or demineralized bones. This organic bone matter, washed
- 20 free of residual acid, is dried for storage or immediately limed. After liming, ossein is subsequently treated as described above for the production of gelatin from bovine hides. In all cases, after final filtering, demineralization, concentration, and drying steps, the resultant gelatin product is divided into batches, subjected to various physical, chemical, and bacteriological tests to determine grade and purity, and ground and blended according to
- 25 commercial requirements. In both type A and B extraction processes, the resultant gelatin product typically comprises a mixture of gelatin molecules, in sizes of from a few thousand up to several hundred thousand Daltons.
- 30 Fish gelatin, classified as gelling or non-gelling types, and typically processed as Type A gelatin, is also used in certain commercial applications. Gelling types are usually derived from the skins of warm water fish, while non-gelling types are typically derived from cold water fish. Fish gelatins have widely varying amino acid compositions, and differ from animal gelatins in having typically lower proportions of proline and hydroxyproline residues. In contrast to animal gelatins, fish gelatins typically remain liquid at much lower
- 35 temperatures, even at comparable average molecular weights. As with other animal gelatins, fish gelatin is extracted by treatment and subsequent hydrolyzation of fish skin. Again, as with animal extraction processes, the process of extracting fish gelatin results in a product that lacks homogeneity.

5

**Gelatin in Vaccines**

Anaphylactic reactions to measles, mumps, and rubella vaccines, and to the combined measles-mumps-rubella (MMR) vaccine, have been reported. (Sakaguchi and Inouye, 2000, *Vaccine*, 18:2055-2058; Sakaguchi *et al.*, 1999, *J Allergy Clin Immunol*, 104:695-699.)

- 10 Despite speculation that these allergic reactions were caused by allergy to egg proteins present in the vaccines, anaphylactic reactions also have been reported to occur after administration of the MMR vaccine in children who tolerated eggs. It has been found that most of the reactions to live vaccines are caused by an acquired sensitivity to the bovine gelatin included in these vaccines. (See, e.g., Nakayama *et al.* (1999) *J Allergy Clin Immunol*
- 15 103:321-325, and references therein; and Sakaguchi *et al.* (1999) *Immunology*, 96:286-290.)

- Studies have revealed that anaphylaxis in children in response to live attenuated viral vaccines containing gelatin is caused by the gelatin, and that gelatin-containing diphtheria-tetanus-acellular pertussis (DTaP) vaccines appear to sensitize children to gelatin. Specifically, a
- 20 strong causal relationship has been identified between DTaP vaccines containing gelatin, anti-gelatin IgE production, and the risk of anaphylaxis following subsequent immunization with live viral vaccines containing bovine gelatin. (Sakaguchi and Inouye, *supra*; Nakayama *et al. supra*.)

- 25 Anaphylactic reactions to MMR vaccines which include gelatin as a stabilizer have been reported and have been shown to be caused by the bovine gelatin included in these vaccines. Specifically, IgE reactivity to  $\alpha 1$  and  $\alpha 2$  chains of bovine type I collagen has been identified in children with bovine gelatin allergy. (Sakaguchi *et al.*, *supra*, and references therein). Numerous anaphylactic reactions and some urticarial reactions to gelatin-containing measles,
- 30 mumps, and rubella vaccines have been associated with IgE-mediated allergenic responses to gelatin. (Nakayama *et al.*, *supra*) Specific IgG antibodies to gelatin have been identified in children with systemic immediate-type and nonimmediate-type reactions to MMR vaccines, suggesting that the immune response to non-human gelatin plays a role in the pathogenesis of systemic reactions to live virus vaccines. (Miyazawa *et al.* (1999) *Vaccine* 17:2176-2180;
- 35 and Kelso (1999) *J Aller. Clin Immunol.* 103:200-202, and references therein.)

Such gelatin-induced vaccine-specific reactions are all the more critical in the context of increasing concern relating to the use of animal-derived, e.g., bovine-derived, materials intended for human and animal use, for example, in pharmaceutical applications, and

5 consumption. Such concerns relating to the safety of bovine-derived materials are directed to the risk of exposure to infectious agents that might survive or be introduced in the process of extraction and purification of gelatin from animal sources. (See, e.g., Asher (1999) *Dev. Biol. Stand.* 99:41-44; and Verdrager (1999) *Lancet* 354:1304-1305.) A certain oral polio vaccine used only in the United Kingdom and the Republic of Ireland was recently withdrawn from  
10 use after it was determined that fetal bovine calf serum from the United Kingdom was used in the manufacture of the vaccine. (BBC News Report, "Polio vaccine in BSE scare," 20 October 2000; World Health Organization, "WHO Position Statement on Recall of Evans/Medeva Polio Vaccine in UK," 20 October 2000.) Concerns over the presence of infective agents, such as TSEs, as well as bacterial and other pathogens and endotoxins which  
15 might exist after extraction, have established a need for safe, non-immunogenic material that can be used in place of the materials currently derived from animal sources.

#### Summary

Current methods of extraction result in a gelatin product that is a heterogeneous mixture of  
20 proteins, containing polypeptides with molecular weight distributions of varying ranges. It is sometimes necessary to blend various lots of product in order to obtain a gelatin mixture with the physical properties appropriate for use in a desired application. In addition, it is virtually impossible, using current extraction methods, to obtain a gelatin free of non-gelatin impurities, e.g., protein, lipid, polysaccharides, etc.

25 A more homogeneous product, and one produced by more reproducible means, would be desirable. The availability of a homogenous material with reproducible physical characteristics would be desirable, for example, in various products and processes, where the availability of gelatin with specific characteristics, such as a fixed range of molecular weight,  
30 would allow for a reproducible and controlled performance. There is thus a need for a reliable and reproducible means of gelatin production that provides a consistent product with controlled characteristics.

In addition, there are concerns relating to the immunogenicity and infectivity of gelatin-  
35 containing products resulting from the animal-source products and methods of their preparation. (See, e.g., Sakaguchi and Inouye, *supra*; Sakaguchi et al., *supra*; Nakayama et al., *supra*; Asher, *supra*; and Verdrager, *supra*.) There is thus a need for a source of gelatin other than that currently extracted from bovine, porcine, and other animal sources.

5     Gelatin producers and end-users have searched for and tested a number of natural and  
synthetic substitutes for the animal-source gelatin currently available. Alternatives have been  
identified for a few applications, such as the use of cellulosic raw materials in VCAPS  
capsules (CAPSUGEL; Morris Plains, NJ), or the proposed use of non-natural gelatin-like  
proteins from mouse and rat collagen sequences in photographic emulsions. (See, e.g.,  
10     Werten, M. W. et al. (1999) Yeast 15:1087-1096; and De Wolf, Anton et al., European  
Application No. EP1014176A2.) However, for most gelatin-based processes and products,  
the performance characteristics of this key material have not been duplicated and substitutes  
have not been adopted. Thus, there is a need for a means of producing gelatin in a synthetic  
and reproducible manner wherein the resultant product can serve as a rational substitute with  
15     the desired performance characteristics.

There is a need for a versatile gelatin product from a non-animal source that is readily  
adaptable for different uses and that answers existing health and special concerns. In  
particular, with respect to vaccines, there is a need for a material that safely minimizes the  
20     risks of immunogenicity, antigenicity, and/or infectivity from the animal-derived products,  
while serving as an effective stabilizer and component of vaccine formulations.

The present invention answers these needs by providing a universal replacement material,  
obtained recombinantly, appropriate for use in the extraordinarily diverse spectrum of  
25     applications in which gelatin is currently used. In particular, the present invention provides  
recombinant gelatin suitable for use in vaccine formulations. The present materials can be  
designed to possess properties and characteristics desired for specific applications, and can  
thus substitute for currently available animal-source gelatins as well as provide new properties  
and uses previously unavailable.

30

#### SUMMARY OF THE INVENTION

The present invention is directed to recombinant gelatins and, specifically, to the use of  
recombinant gelatins in vaccines. Therefore, in one embodiment, the present invention  
provides a vaccine formulation comprising recombinant gelatin. In a preferred embodiment,  
35     the recombinant gelatin is human gelatin. A further aspect of the present invention provides  
that the recombinant gelatin is non-immunogenic.

In one embodiment, the present invention provides a vaccine formulation comprising  
recombinant gelatin, wherein the recombinant gelatin confers stability at ambient

5 temperatures. In another embodiment, the gelatin is derived from non-native collagen sequence.

Formulations comprising specific recombinant gelatins are contemplated. In one aspect, the recombinant gelatin has a molecular weight range selected from the group consisting of about 0  
10 to 50 kDa, about 10 to 30 kDa, about 30 to 50 kDa, about 10 to 70 kDa, about 50 kDa to 70 kDa  
about 50 to 100 kDa, about 100 to 150 kDa, about 150 to 200 kDa, about 200 to 250 kDa, about  
250 to 300 kDa, and about 300 to 350 kDa. In certain embodiments, the recombinant gelatin has  
a molecular weight selected from the group consisting of about 1 kDa, about 5 kDa, about 8  
kDa, about 9 kDa, about 14 kDa, about 16 kDa, about 22 kDa, about 23 kDa, about 44 kDa, and  
15 about 65 kDa.

The present invention provides, in one embodiment, a vaccine formulation comprising recombinant gelatin derived from one collagen free of any other type of collagen. In various aspects, a vaccine formulation comprising recombinant gelatin is provided, wherein the  
20 recombinant gelatin is produced by processing of recombinant collagen, or is produced directly from an altered collagen construct.

In specific embodiments, the present invention encompasses vaccine formulations comprising a sequence selected from the group consisting of SEQ ID NOs:15 through 25, 30, 31,  
25 and 33.

The vaccine formulations of the present invention can be suitable for various modes of delivery, including delivery by injection, nasal delivery, oral delivery, transdermal delivery, and deep lung delivery. The vaccine formulations of the present invention can be formulated in a number of  
30 ways, for example, in liquid, dry, powdered, spray, and inhalant form. In various embodiments, the present vaccine formulations can comprise live, inactivated, subunit, single dosage, multiple dosage, conjugate, nucleic acid, DNA, combined, and acellular vaccines. Specific vaccines are contemplated, including, but not limited to, vaccines formulated for the prevention of a disease selected from the group consisting of vaccinia virus (small pox), polio virus (Salk and Sabin),  
35 mumps, measles, rubella, diphtheria, tetanus, Varicella-Zoster (chicken pox/shingles), pertussis (whooping cough), Bacille Calmette-Guerin (BCG, tuberculosis), haemophilus influenzae meningitis, rabies, cholera, Japanese encephalitis virus, salmonella typhi, shigella, hepatitis A, hepatitis B, adenovirus, yellow fever, foot-and-mouth disease, herpes simplex virus, respiratory



- 5 syncytial virus, rotavirus, Dengue, West Nile virus, Turkey herpes virus (Marek's Disease), influenza, and anthrax.

In some embodiments, the present invention provides vaccine formulations comprising recombinant gelatin, wherein the recombinant gelatin has endotoxin levels below 1.000 EU/mg,  
10 0.500 EU/mg, 0.050 EU/mg, and 0.005 EU/mg. In one aspect, the present invention encompasses a vaccine formulation comprising recombinant gelatin wherein the recombinant gelatin is proteolytically stable. A vaccine stabilizer comprising recombinant gelatin is specifically contemplated. The present invention provides methods for producing vaccine formulations comprising recombinant gelatin. In one embodiment, the method comprises  
15 providing recombinant gelatin and providing a vaccine, and combining the recombinant gelatin and the vaccine. A method of inducing an immune response in a subject is also provided, the method comprising administering a vaccine comprising recombinant gelatin to the subject. Further, kits comprising a vaccine comprising recombinant gelatin and a delivery device for the vaccine are also contemplated. In various embodiments, the delivery device is a device suitable,  
20 for example, for injectable, nasal, mucosal, and aerosol delivery.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 sets forth results showing the expression of recombinant gelatins.  
25

Figures 2A and 2B set forth results demonstrating that recombinant gelatins support cell attachment.

Figure 3 sets forth results demonstrating the production of proteolytically stable recombinant gelatins.  
30

Figure 4 sets forth results demonstrating the production of hydroxylated recombinant gelatins.

Figure 5 sets forth results showing the purification of recombinant gelatin following *in vitro* hydroxylation.  
35

Figure 6 sets forth results showing the stability of recombinant gelatins expressed in the presence or absence of prolyl 4-hydroxylase.

- 5     Figure 7 sets forth results demonstrating enhanced recombinant gelatin expression by supplementation of expression media

Figure 8 sets forth results comparing commercially available gelatins to cross-linked recombinant gelatin.

10

Figure 9 sets forth results comparing the molecular weight distribution of commercially available gelatins.

- 15     Figures 10A, 10B, 10C, 10D, 10E, and 10F set forth results showing the hydrolysis of commercially available gelatins performed at 120°C.

Figures 11A, 11B, 11C, and 11D set forth results showing the hydrolysis of commercially available gelatins performed at 150°C.

- 20     Figures 12A and 12B set forth results showing the acid and thermal hydrolysis of recombinant human collagen type I and type III.

Figure 13 sets forth results showing the enzymatic hydrolysis of recombinant human collagen type I.

25

Figure 14 sets forth a Western blot analysis of recombinant human collagens and recombinant human gelatins using antisera from Guinea pigs immunized with recombinant human collagen type I.

- 30     Figures 15A and 15B set forth results showing antisera from Guinea pigs immunized with recombinant human collagen type I is reactive to specific cyanogen bromide fragments of collagen type I.

- 35     Figure 16 sets forth ELISA results showing antisera from Guinea pigs immunized with recombinant human collagen type I is not reactive to recombinant human gelatins.

5

**DESCRIPTION OF THE INVENTION**

Before the present proteins, nucleotide sequences, and methods are described, it is understood that this invention is not limited to the particular methodology, protocols, cell lines, vectors, and reagents described, as these may vary. It is also to be understood that the terminology  
10 used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention.

It must be noted that as used herein, and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for  
15 example, reference to "a host cell" is reference to one or more of such host cells and equivalents thereof known to those skilled in the art, and reference to "an antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

20 Unless defined otherwise, all technical and scientific terms used herein have the meanings as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by  
25 reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies, etc., which are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention. Each reference cited herein is incorporated herein by reference in its entirety.

30

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Gennaro, A.R., ed. (1990) Remington's Pharmaceutical Sciences, 18<sup>th</sup> ed., Mack Publishing  
35 Co.; Colowick, S. et al., eds., Methods In Enzymology, Academic Press, Inc.; Handbook of Experimental Immunology, Vols. I-IV (D.M. Weir and C.C. Blackwell, eds., 1986, Blackwell Scientific Publications); Maniatis, T. et al., eds. (1989) Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> edition, Vols. I-III, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al., eds. (1999) Short Protocols in Molecular Biology, 4<sup>th</sup> edition, John Wiley & Sons; Ream et

- 5 al., eds. (1998) *Molecular Biology Techniques: An Intensive Laboratory Course*, Academic Press); PCR (Introduction to Biotechniques Series), 2nd ed. (Newton & Graham eds., 1997, Springer Verlag).

#### DEFINITIONS

- 10 The term "collagen" refers to any one of the known collagen types, including collagen types I through XX, as well as to any other collagens, whether natural, synthetic, semi-synthetic, or recombinant. The term also encompasses procollagens. The term collagen encompasses any single-chain polypeptide encoded by a single polynucleotide, as well as homotrimeric and heterotrimeric assemblies of collagen chains. The term "collagen" specifically encompasses  
15 variants and fragments thereof, and functional equivalents and derivatives thereof, which preferably retain at least one structural or functional characteristic of collagen, for example, a (Gly-X-Y)<sub>n</sub> domain.

- The term "procollagen" refers to a procollagen corresponding to any one of the collagen types I through XX, as well as to a procollagen corresponding to any other collagens, whether natural,  
20 synthetic, semi-synthetic, or recombinant, that possesses additional C-terminal and/or N-terminal propeptides or telopeptides that assist in trimer assembly, solubility, purification, or any other function, and that then are subsequently cleaved by N-proteinase, C-proteinase, or other enzymes, e.g., proteolytic enzymes, associated with collagen production. The term procollagen  
25 specifically encompasses variants and fragments thereof, and functional equivalents and derivatives thereof, which preferably retain at least one structural or functional characteristic of collagen, for example, a (Gly-X-Y)<sub>n</sub> domain.

- "Gelatin" as used herein refers to any gelatin, whether extracted by traditional methods or  
30 recombinant or biosynthetic in origin, or to any molecule having at least one structural and/or functional characteristic of gelatin. Gelatin is currently obtained by extraction from collagen derived from animal (e.g., bovine, porcine, rodent, chicken, equine, piscine, etc.) sources, for example, bones and tissues. The term gelatin encompasses both the composition of more than one polypeptide included in a gelatin product, as well as an individual polypeptide  
35 contributing to the gelatin material. Thus, the term recombinant gelatin as used in reference to the present invention encompasses both a recombinant gelatin material comprising the present gelatin polypeptides, as well as an individual gelatin polypeptide of the present invention.

- 5 Polypeptides from which gelatin can be derived are polypeptides such as collagens, procollagens, and other polypeptides having at least one structural and/or functional characteristic of collagen. Such a polypeptide could include a single collagen chain, or a collagen homotrimer or heterotrimer, or any fragments, derivatives, oligomers, polymers, or subunits thereof, containing at least one collagenous domain (a Gly-X-Y region). The term
- 10 specifically contemplates engineered sequences not found in nature, such as altered collagen constructs, etc. An altered collagen construct is a polynucleotide comprising a sequence that is altered, through deletions, additions, substitutions, or other changes, from the naturally occurring collagen gene.
- 15 An "adjuvant" is any agent added to a drug or vaccine to increase, improve, or otherwise aid its effect. An adjuvant used in a vaccine formulation might be an immunological agent that improves the immune response by producing a non-specific stimulator of the immune response. Adjuvants are often used in non-living vaccines.
- 20 The terms "allele" or "allelic sequence" refer to alternative forms of genetic sequences. Alleles may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or polypeptides whose structure or function may or may not be altered. Any given natural or recombinant gene may have none, one, or many allelic forms. Common mutational changes which give rise to alleles are generally ascribed to natural deletions, additions, or
- 25 substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.
- "Altered" polynucleotide sequences include those with deletions, insertions, or substitutions of different nucleotides resulting in a polynucleotide that encodes the same or a functionally
- 30 equivalent polypeptide. Included within this definition are sequences displaying polymorphisms that may or may not be readily detectable using particular oligonucleotide probes or through deletion of improper or unexpected hybridization to alleles, with a locus other than the normal chromosomal locus for the subject polynucleotide sequence.
- 35 "Altered" polypeptides may contain deletions, insertions, or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent polypeptide. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the biological or immunological activity of the encoded polypeptide is retained. For example,

- 5 negatively charged amino acids may include aspartic acid and glutamic acid; positively charged amino acids may include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values may include leucine, isoleucine, and valine, glycine and alanine, asparagine and glutamine, serine and threonine, and phenylalanine and tyrosine.
- 10 "Amino acid" or "polypeptide" sequences or "polypeptides," as these terms are used herein, refer to oligopeptide, peptide, polypeptide, or protein sequences, and fragments thereof, and to naturally occurring or synthetic molecules. Polypeptide or amino acid fragments are any portion of a polypeptide which retains at least one structural and/or functional characteristic of the polypeptide. In at least one embodiment of the present invention, polypeptide fragments are
- 15 those retaining at least one (Gly-X-Y)<sub>n</sub> region.

The term "animal" as it is used in reference, for example, to "animal collagens," encompasses any collagens, derived from animal sources, whether natural, synthetic, semi-synthetic, or recombinant. Animal sources include, for example, mammalian sources, including, but not

20 limited to, bovine, porcine, rodent, equine, and ovine sources, and other animal sources, including, but not limited to, chicken and piscine sources, and non-vertebrate sources.

"Antigenicity" relates to the ability of a substance to, when introduced into the body, stimulate the immune response and the production of an antibody. An agent displaying the

25 property of antigenicity is referred to as being antigenic. Antigenic agents can include, but are not limited to, a variety of macromolecules such as, for example, proteins, lipoproteins, polysaccharides, nucleic acids, bacteria and bacterial components, and viruses and viral components.

30 The terms "complementary" or "complementarity," as used herein, refer to the natural binding of polynucleotides by base-pairing. For example, the sequence "A-G-T" binds to the complementary sequence "T-C-A." Complementarity between two single-stranded molecules may be "partial," when only some of the nucleic acids bind, or may be complete, when total complementarity exists between the single stranded molecules. The degree of complementarity

35 between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, which depend upon binding between nucleic acids strands, and in the design and use, for example, of peptide nucleic acid (PNA) molecules.

- 5 A "deletion" is a change in an amino acid or nucleotide sequence that results in the absence of one or more amino acid residues or nucleotides.

The term "derivative," as applied to polynucleotides, refers to the chemical modification of a polynucleotide encoding a particular polypeptide or complementary to a polynucleotide  
10 encoding a particular polypeptide. Such modifications include, for example, replacement of hydrogen by an alkyl, acyl, or amino group. As used herein to refer to polypeptides, the term "derivative" refers to a polypeptide which is modified, for example, by hydroxylation, glycosylation, pegylation, or by any similar process. The term "derivatives" encompasses those molecules containing at least one structural and/or functional characteristic of the  
15 molecule from which it is derived.

A molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties can improve the molecule's solubility, absorption, biological half-life, and the like. The moieties can  
20 alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, and the like. Moieties capable of mediating such effects are generally available in the art and can be found for example, in Remington's Pharmaceutical Sciences, *supra*. Procedures for coupling such moieties to a molecule are well known in the art.

- 25 An "excipient" as the term is used herein is any inert substance used as a diluent or vehicle in the formulation of a drug, a vaccine, or other pharmaceutical composition, in order to confer a suitable consistency or form to the drug, vaccine, or pharmaceutical composition.

The term "functional equivalent" as it is used herein refers to a polypeptide or polynucleotide  
30 that possesses at least one functional and/or structural characteristic of a particular polypeptide or polynucleotide. A functional equivalent may contain modifications that enable the performance of a specific function. The term "functional equivalent" is intended to include fragments, mutants, hybrids, variants, analogs, or chemical derivatives of a molecule.

- 35 A "fusion protein" is a protein in which peptide sequences from different proteins are operably linked.

The term "hybridization" refers to the process by which a nucleic acid sequence binds to a complementary sequence through base pairing. Hybridization conditions can be defined by, for

5     example, the concentrations of salt or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and are well known in the art. Hybridization can occur under conditions of various stringency.

10     In particular, stringency can be increased by reducing the concentration of salt, increasing the concentration of formamide, or raising the hybridization temperature. For example, for purposes of the present invention, hybridization under high stringency conditions occurs in about 50% formamide at about 37°C to 42°C, and under reduced stringency conditions in about 35% to 25% formamide at about 30°C to 35°C. In particular, hybridization occurs in conditions of highest stringency at 42°C in 50% formamide, 5X SSPE, 0.3% SDS, and 200 µg/ml sheared and  
15     denatured salmon sperm DNA.

20     The temperature range corresponding to a particular level of stringency can be further narrowed by methods known in the art, for example, by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. To remove nonspecific signals, blots can be sequentially washed, for example, at room temperature under increasingly stringent conditions of up to 0.1X SSC and 0.5% SDS. Variations on the above ranges and conditions are well known in the art.

25     “Immunogenicity” relates to the ability to evoke an immune response within an organism. An agent displaying the property of immunogenicity is referred to as being immunogenic. Agents can include, but are not limited to, a variety of macromolecules such as, for example, proteins, lipoproteins, polysaccharides, nucleic acids, bacteria and bacterial components, and viruses and viral components. Immunogenic agents often have a fairly high molecular weight (usually greater than 10 kDa).

30     “Infectivity” refers to the ability to be infective or the ability to produce infection, referring to the invasion and multiplication of microorganisms, such as bacteria or viruses within the body.

35     The terms “insertion” or “addition” refer to a change in a polypeptide or polynucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively, as compared to the naturally occurring molecule.



5     The term "isolated" as used herein refers to a molecule separated not only from proteins, etc., that are present in the natural source of the protein, but also from other components in general, and preferably refers to a molecule found in the presence of, if anything, only a solvent, buffer, ion, or other component normally present in a solution of the same. As used herein, the terms "isolated" and "purified" do not encompass molecules present in their natural source.

10

      The term "microarray" refers to any arrangement of nucleic acids, amino acids, antibodies, etc., on a substrate. The substrate can be any suitable support, e.g., beads, glass, paper, nitrocellulose, nylon, or any appropriate membrane, etc. A substrate can be any rigid or semi-rigid support including, but not limited to, membranes, filters, wafers, chips, slides, fibers, beads, including  
15    magnetic or nonmagnetic beads, gels, tubing, plates, polymers, microparticles, capillaries, etc. The substrate can provide a surface for coating and/or can have a variety of surface forms, such as wells, pins, trenches, channels, and pores, to which the nucleic acids, amino acids, etc., may be bound.

20    The term "microorganism" can include, but is not limited to, viruses, bacteria, Chlamydia, rickettsias, mycoplasmas, ureaplasmas, fungi, and parasites, including infectious parasites such as protozoans.

      The terms "nucleic acid" or "polynucleotide" sequences or "polynucleotides" refer to  
25    oligonucleotides, nucleotides, or polynucleotides, or any fragments thereof, and to DNA or RNA of natural or synthetic origin which may be single- or double-stranded and may represent the sense or antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin. Polynucleotide fragments are any portion of a polynucleotide sequence that retains at least one structural or functional characteristic of the  
30    polynucleotide. In one embodiment of the present invention, polynucleotide fragments are those that encode at least one (Gly-X-Y)<sub>n</sub> region. Polynucleotide fragments can be of variable length, for example, greater than 60 nucleotides in length, at least 100 nucleotides in length, at least 1000 nucleotides in length, or at least 10,000 nucleotides in length.

35    The phrase "percent similarity" (% similarity) refers to the percentage of sequence similarity found in a comparison of two or more polypeptide or polynucleotide sequences. Percent similarity can be determined by methods well-known in the art. For example, percent similarity between amino acid sequences can be calculated using the Clustal method. (See, e.g., Higgins, D. G. and P. M. Sharp (1988) *Gene* 73:237-244.) The Clustal algorithm groups

- 5 sequences into clusters by examining the distances between all pairs. The clusters are aligned pairwise and then in groups. The percentage similarity between two amino acid sequences, e.g., sequence A and sequence B, is calculated by dividing the length of sequence A, minus the number of gap residues in sequence A, minus the number of gap residues in sequence B, into the sum of the residue matches between sequence A and sequence B, times one hundred.
- 10 Gaps of low or of no homology between the two amino acid sequences are not included in determining percentage similarity. Percent similarity can be calculated by other methods known in the art, for example, by varying hybridization conditions, and can be calculated electronically using programs such as the MEGALIGN program (DNASTAR Inc., Madison, Wisconsin).

15

- As used herein, the term "plant" includes reference to one or more plants, i.e., any eukaryotic autotrophic organisms, such as angiosperms and gymnosperms, monocotyledons and dicotyledons, etc., including, but not limited to, soybean, cotton, alfalfa, flax, tomato, sugar, beet, sunflower, potato, tobacco, maize, wheat, rice, lettuce, banana, cassava, safflower,
- 20 oilseed, rape, mustard, canola, hemp, algae, kelp, etc. The term "plant" also encompasses one or more plant cells. The term "plant cells" includes, but is not limited to, vegetative tissues and organs such as seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, tubers, corms, bulbs, flowers, fruits, cones, microspores, etc.

25

- The term "post-translational enzyme" refers to any enzyme that catalyzes post-translational modification of, for example, any collagen or procollagen. The term encompasses, but is not limited to, for example, prolyl hydroxylase, peptidyl prolyl isomerase, collagen galactosyl hydroxyllysyl glucosyl transferase, hydroxyllysyl galactosyl transferase, C-proteinase, N-proteinase, lysyl hydroxylase, and lysyl oxidase.
- 30

- As used herein, the term "promoter" generally refers to a regulatory region of nucleic acid sequence capable of initiating, directing, and mediating the transcription of a polynucleotide sequence. Promoters may additionally comprise recognition sequences, such as upstream or
- 35 downstream promoter elements, which may influence the transcription rate.

The term "non-constitutive promoters" refers to promoters that induce transcription via a specific tissue, or may be otherwise under environmental or developmental controls, and includes repressible and inducible promoters such as tissue-preferred, tissue-specific, and cell

- 5     type-specific promoters. Such promoters include, but are not limited to, the AdH1 promoter, inducible by hypoxia or cold stress, the Hsp70 promoter, inducible by heat stress, and the PPDk promoter, inducible by light.

Promoters which are "tissue-preferred" are promoters that preferentially initiate transcription  
10     in certain tissues. Promoters which are "tissue-specific" are promoters that initiate transcription only in certain tissues. "Cell type-specific" promoters are promoters which primarily drive expression in certain cell types in at least one organ, for example, vascular cells.

- 15     "Inducible" or "repressible" promoters are those under control of the environment, such that transcription is effected, for example, by an environmental condition such as anaerobic conditions, the presence of light, biotic stresses, etc., or in response to internal, chemical, or biological signals, e.g., glyceraldehyde phosphate dehydrogenase, AOX1 and AOX2 methanol-inducible promoters, or to physical damage.

20     As used herein, the term "constitutive promoters" refers to promoters that initiate, direct, or mediate transcription, and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters, include, but are not limited to, the cauliflower mosaic virus (CaMv) 35S, the 1'- or 2'- promoter derived from T-  
25     DNA of *Agrobacterium tumefaciens*, the ubiquitin 1 promoter, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter, glyceraldehyde dehydrogenase promoter, and the Nos promoter, etc.

30     The term "purified" as it is used herein denotes that the indicated molecule is present in the substantial absence of other biological macromolecules, e.g., polynucleotides, proteins, and the like. The term preferably contemplates that the molecule of interest is present in a solution or composition at least 80% by weight; preferably, at least 85% by weight; more preferably, at least 95% by weight; and, most preferably, at least 99.8% by weight. Water, buffers, and other small molecules, especially molecules having a molecular weight of less  
35     than about one kDa, can be present.

The term "substantially purified", as used herein, refers to nucleic or amino acid sequences that are removed from their natural environment, isolated or separated, and are at least 60%

- 5 free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated.

A "substitution" is the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

10

The term "transfection" as used herein refers to the process of introducing an expression vector into a cell. Various transfection techniques are known in the art, for example, microinjection, lipofection, or the use of a gene gun.

- 15 "Transformation", as defined herein, describes a process by which exogenous nucleic acid sequences, e.g., DNA, enters and changes a recipient cell. Transformation may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is selected based on the type of host cell being transformed
- 20 and may include, but is not limited to, viral infection, electroporation, heat shock, lipofection, and particle bombardment. Such "transformed" cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome, and also include cells which transiently express the inserted nucleic acid for limited periods of time.

25

- As used herein, the term "vaccine" refers to a preparation of killed or modified microorganisms, living attenuated organisms, or living fully virulent organisms, or any other agents, including, but not limited to peptides, proteins, biological macromolecules, or nucleic acids, natural, synthetic, or semi-synthetic, administered to produce or artificially increase
- 30 immunity to a particular disease, in order to prevent future infection with a similar entity. Vaccines can contain live or inactivated microorganisms or agents, including viruses and bacteria, as well as subunit, synthetic, semi-synthetic, or recombinant DNA-based.

- Vaccines can be monovalent (a single strain/microorganism/disease vaccine) consisting of
- 35 one microorganism or agent (e.g., poliovirus vaccine) or the antigens of one microorganism or agent. Vaccines can also be multivalent, e.g., divalent, trivalent, etc. (a combined vaccine), consisting of more than one microorganism or agent (e.g., a measles-mumps-rubella (MMR) vaccine) or the antigens of more than one microorganism or agent.

5 Live vaccines are prepared from living microorganisms. Attenuated vaccines are live  
vaccines prepared from microorganisms which have undergone physical alteration (such as  
radiation or temperature conditioning) or serial passage in laboratory animal hosts or infected  
tissue/cell cultures, such treatments producing avirulent strains or strains of reduced  
virulence, but maintaining the capability of inducing protective immunity. Examples of live  
10 attenuated vaccines include measles, mumps, rubella, and canine distemper. Inactivated  
vaccines are vaccines in which the infectious microbial components have been destroyed, e.g.,  
by chemical or physical treatment (such as formalin, beta-propiolactone, or gamma radiation),  
without affecting the antigenicity or immunogenicity of the viral coat or bacterial outer  
membrane proteins. Examples of inactivated or subunit vaccines include influenza, Hepatitis  
15 A, and poliomyelitis (IPV) vaccines.

Subunit vaccines are composed of key macromolecules from, e.g., the viral, bacterial, or other  
agent responsible for eliciting an immune response. These components can be obtained in a  
number of ways, for example, through purification from microorganisms, generation using  
20 recombinant DNA technology, etc. Subunit vaccines can contain synthetic mimics of any  
infective agent. Subunit vaccines can include macromolecules such as bacterial protein toxins  
(e.g., tetanus, diphtheria), viral proteins (e.g., from influenza virus), polysaccharides from  
encapsulated bacteria (e.g., from *Haemophilus influenzae* and *Streptococcus pneumoniae*), and  
viruslike particles produced by recombinant DNA technology (e.g., hepatitis B surface  
25 antigen), etc.

Synthetic vaccines are vaccines made up of small synthetic peptides that mimic the surface  
antigens of pathogens and are immunogenic, or may be vaccines manufactured with the aid of  
recombinant DNA techniques, including whole viruses whose nucleic acids have been  
30 modified.

Semi-synthetic vaccines, or conjugate vaccines, consist of polysaccharide antigens from  
microorganisms attached to protein carrier molecules.

35 DNA vaccines contain recombinant DNA vectors encoding antigens, which, upon expression  
of the encoded antigen in host cells having taken up the DNA, induce humoral and cellular  
immune responses against the encoded antigens.

- 5 Vaccines have been developed for a variety of infectious agents. The present invention is directed to recombinant gelatins that can be used in vaccine formulations regardless of the agent involved, and are thus not limited to use in the vaccines specifically described herein by way of example. Vaccines include, but are not limited to, vaccines for vaccinia virus (small pox), polio virus (Salk and Sabin), mumps, measles, rubella, diphtheria, tetanus, Varicella-  
10 Zoster (chicken pox/shingles), pertussis (whooping cough), Bacille Calmette-Guerin (BCG, tuberculosis), haemophilus influenzae meningitis, rabies, cholera, Japanese encephalitis virus, salmonella typhi, shigella, hepatitis A, hepatitis B, adenovirus, yellow fever, foot-and-mouth disease, herpes simplex virus, respiratory syncytial virus, rotavirus, Dengue, West Nile virus, Turkey herpes virus (Marek's Disease), influenza, and anthrax. The term vaccine as used  
15 herein includes reference to vaccines to various infectious and autoimmune diseases and cancers that have been or that will be developed, for example, vaccines to various infectious and autoimmune diseases and cancers, e.g., vaccines to HIV, HCV, malaria, and vaccines to breast, lung, colon, renal, bladder, and ovarian cancers.
- 20 A polypeptide or amino acid "variant" is an amino acid sequence that is altered by one or more amino acids from a particular amino acid sequence. A polypeptide variant may have conservative changes, wherein a substituted amino acid has similar structural or chemical properties to the amino acid replaced, e.g., replacement of leucine with isoleucine. A variant may also have nonconservative changes, in which the substituted amino acid has physical  
25 properties different from those of the replaced amino acid, e.g., replacement of a glycine with a tryptophan. Analogous minor variations may also include amino acid deletions or insertions, or both. Preferably, amino acid variants retain certain structural or functional characteristics of a particular polypeptide. Guidance in determining which amino acid residues may be substituted, inserted, or deleted may be found, for example, using computer programs well known in the art,  
30 such as LASERGENE software (DNASTAR Inc., Madison, WI).

A polynucleotide variant is a variant of a particular polynucleotide sequence that preferably has at least about 80%, more preferably at least about 90%, and most preferably at least about 95% polynucleotide sequence similarity to the particular polynucleotide sequence. It will be  
35 appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of variant polynucleotide sequences encoding a particular protein, some bearing minimal homology to the polynucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention contemplates each and every possible variation of polynucleotide sequence that could be made by selecting combinations based on possible

- 5 codon choices. These combinations are made in accordance with the standard codon triplet genetic code, and all such variations are to be considered as being specifically disclosed.

#### INVENTION

- The present invention provides recombinant gelatins and methods for producing these  
10 gelatins. The recombinant gelatins of the present invention provide reproducible and improved performance, and answer various health and other concerns. Using the present methods, gelatin can be directly manufactured, rather than extracted from animal sources through lengthy and harsh processes. The recombinant gelatin of the present invention is free of pathogens, for example, pathogenic bacteria, transmissible spongiform encephalopathies  
15 (TSEs), etc. The present methods minimize variability and allow for a degree of reproducibility unattainable in current extraction methods.

- Safety issues, such as concern over potential immunogenic, e.g., antigenic and allergenic, responses, have arisen regarding the use of animal-derived products. The inability to  
20 completely characterize, purify, or reproduce animal-source gelatin mixtures used currently is of ongoing concern in the pharmaceutical and medical communities. Additional safety concerns exist with respect to bacterial contamination and endotoxin loads resulting from the extraction and purification processes.

- 25 The recombinant gelatins of the present invention address these concerns as they are virtually free of bacterial contamination or endotoxins. Furthermore, the recombinant human gelatins of the present invention will offer distinct advantages over animal-derived counterparts currently in use, as the use of gelatins derived from native human sequence can eliminate the risk of immune response due to the use of non-human, animal-derived proteins.

- 30 In addition, the present gelatins can be produced as various and distinct materials, with characteristics optimized for particular applications. The resultant products are internally more consistent and uniform than are currently available gelatins derived from animal sources.

- 35 In one embodiment, the present invention provides a recombinant gelatin. The gelatin can be produced using sequences from various species including, but not limited to, human, bovine, porcine, equine, and piscine species. The gelatin of the present invention has increased purity as compared to the gelatin products of current methods of manufacture, and has a reduced

- 5 protein load and reduced levels of endotoxins and other contaminants, including nucleic acids, polysaccharides, prions, etc. The present gelatin is thus safer to use than gelatin manufactured by current methods, and can be administered to or ingested by humans and animals at a higher dosage while minimizing the risk of negative side effects.
- 10 The gelatins of the present invention have increased activity and workability compared to commercial gelatins, as the present gelatin can be produced directly with characteristics optimized for specific uses, improving one's ability to use and formulate the gelatin. While gelatins currently extracted from animal sources are heterogeneous products with a wide range in molecular weights throughout a given batch or sample, the gelatins of the present
- 15 invention include consistent, homogeneous, and reproducible products.

The recombinant gelatins of the present invention can be produced in a variety of methods. In one method, the recombinant gelatin is produced through processing of recombinant collagen. (See, e.g., Examples 7, 10, and 11.) In another method, the recombinant gelatin is produced

20 directly from the expression of altered collagen constructs, i.e., constructs containing a polynucleotide encoding at least one collagenous domain, but not encoding naturally occurring collagen. (See, e.g., Examples 1, 4, and 6.) In another aspect, the recombinant gelatin is derived from polypeptides which are not full-length naturally occurring collagen or procollagen, but which contain at least one collagenous domain. (See, e.g., SEQ ID NOs:15

25 through 25, 30, 31, and 33.) Recombinant gelatins can also comprise sequences containing additional N-terminal or C-terminal propeptides. (See, e.g., SEQ ID NOs:26 through 29.)

In one aspect, the recombinant gelatin of the present invention is derived from recombinant collagens or procollagens. Collagen molecules generally result from trimeric assembly of

30 polypeptide chains containing (Gly-X-Y)<sub>n</sub> repeats which allow for the formation of triple helical domains under normal biological conditions. (See, e.g., van der Rest et al., (1991), FASEB J. 5:2814-2823.) At present, about twenty distinct collagen types have been identified in vertebrates, including bovine, ovine, porcine, chicken and human collagens. A detailed description of structure and biological functions of the various types of naturally

35 occurring collagens can be found, for example, in Ayad et al., The Extracellular Matrix Facts Book, Academic Press, San Diego, CA; Burgeson, R. E., and Nimmi (1992) "Collagen types: Molecular Structure and Tissue Distribution," Clin. Orthop. 282:250-272; Kielty, C. M. et al. (1993) "The Collagen Family: Structure, Assembly And Organization In The Extracellular Matrix," in Connective Tissue And Its Heritable Disorders, Molecular Genetics, And Medical



- 5 Aspects, Royce, P. M. and Steinmann, B., Eds., Wiley-Liss, NY, pp. 103-147; and Prockop and Kivirikko (1995) "Collagens: Molecular biology, diseases, and potentials for therapy", *Annu Rev Biochem* 64:403-434.

10 Type I collagen is the major fibrillar collagen of bone and skin, comprising approximately 80-90% of an organism's total collagen. Type I collagen is the major structural macromolecule present in the extracellular matrix of multicellular organisms and comprises approximately 20% of total protein mass. Type I collagen is a heterotrimeric molecule comprising two  $\alpha 1(I)$  chains and one  $\alpha 2(I)$  chain, which are encoded by the COL1A1 and COL1A2 genes, respectively. Other collagen types are less abundant than type I collagen and exhibit different  
15 distribution patterns. For example, type II collagen is the predominant collagen in cartilage and vitreous humor, while type III collagen is found at high levels in blood vessels and to a lesser extent in skin.

20 Type III collagen is a major fibrillar collagen found in skin and vascular tissues. Type III collagen is a homotrimeric collagen comprising three identical  $\alpha 1(III)$  chains encoded by the COL3A1 gene. Methods for purifying various collagens from tissues can be found, for example, in, Byers et al. (1974) *Biochemistry* 13:5243-5248; and Miller and Rhodes (1982) *Methods in Enzymology* 82:33-64.

25 Post-translational enzymes are important to the biosynthesis of procollagens and collagens. For example, prolyl 4-hydroxylase is a post-translational enzyme necessary for the synthesis of procollagen or collagen by cells. This enzyme hydroxylates prolyl residues in the Y-position of repeating Gly-X-Y sequences to 4-hydroxyproline. (See, e.g., Prockop et al. (1984) *N. Engl. J. Med.* 311:376-386.) Unless an appropriate number of Y-position prolyl  
30 residues are hydroxylated to 4-hydroxyproline by prolyl 4-hydroxylase, the newly synthesized chains cannot maintain a stable triple-helical conformation. Moreover, if no hydroxylation or under-hydroxylation occurs, the polypeptides are not secreted properly and may be degenerated.

35 Vertebrate prolyl 4-hydroxylase is an  $\alpha_2\beta_2$  tetramer. (See, e.g. Berg and Prockop (1973) *J. Biol. Chem.* 248:1175-1192; and Tuderman et al. (1975) *Eur. J. Biochem.* 52:9-16.) The  $\alpha$  subunits contain the catalytic sites involved in the hydroxylation of prolyl residues, but are insoluble in the absence of  $\beta$  subunits. The  $\beta$  subunits, protein disulfide isomerases, catalyze

5     thiol/disulfide interchanges, leading to formation of disulfide bonds essential to establishing a stable protein. The  $\beta$  subunits retain 50% of protein disulfide isomerase activity when part of the prolyl 4-hydroxylase tetramer. (See, e.g., Pihlajaniemi et al. (1987) *Embo J.* 6:643-649; Parkkonen et al. (1988) *Biochem. J.* 256:1005-1011; and Koivu et al. (1987) *J. Biol. Chem.* 262:6447-6449.)

10

Active recombinant human prolyl 4-hydroxylase has been produced in, e.g., Sf9 insect cells and in yeast cells, by simultaneously expressing the  $\alpha$  and  $\beta$  subunits. (See, e.g., Vuori et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7467-7470; U.S. Patent No. 5, 593,859.) In addition to prolyl 4-hydroxylase, other collagen post-translational enzymes have been identified and  
15     reported in the literature, including C-proteinase, N-proteinase, lysyl oxidase, lysyl hydroxylase, etc. (See, e.g., Olsen et al. (1991) Cell Biology of Extracellular Matrix, 2<sup>nd</sup> ed., Hay editor, Plenum Press, New York.)

The present invention specifically contemplates the use of any compound, biological or  
20     chemical, that confers hydroxylation, e.g., proline hydroxylation and/or lysyl hydroxylation, etc., as desired, to the present recombinant gelatins. This includes, for example, prolyl 4-hydroxylase from any species, endogenously or exogenously supplied, including various isoforms of prolyl 4-hydroxylase and any variants or fragments or subunits of prolyl 4-hydroxylase having the desired activity, whether native, synthetic, or semi-synthetic, and  
25     other hydroxylases such as prolyl 3-hydroxylase, etc. (See, e.g., U.S. Patent No. 5,928,922, incorporated by reference herein in its entirety.) In one embodiment, the prolyl hydroxylase activity is conferred by a prolyl hydroxylase derived from the same species as the polynucleotide encoding recombinant gelatin or encoding a polypeptide from which recombinant gelatin can be derived. In a further embodiment, the prolyl 4-hydroxylase is  
30     human and the encoding polynucleotide is derived from human sequence.

The present invention provides methods for manipulating the thermoplasticity of gelatin in order to produce a material with the desired physical characteristics. In one method, the encoding polynucleotides are expressed in a host system having endogenous prolyl  
35     hydroxylase or alternate hydroxylases, such as certain mammalian or insect cells, or transgenic animals, or plants or plant cells. In such a system, the present invention provides methods for producing a mixture of recombinant gelatins having a range of percentages of hydroxylation, i.e., non-hydroxylated, partially hydroxylated, and fully hydroxylated portions. For example, in one method of producing recombinant gelatins with varying percentages of

5 hydroxylation, the hydroxylation is conferred by endogenous prolyl hydroxylase in, e.g., a transgenic animal, and the distribution of percentage hydroxylation ranges from non-hydroxylated to fully-hydroxylated, and the melting temperatures of the material produced range from 28°C to 36°C, with a median  $T_m$  value of around 30°C to 32°C. If desired, different fractions of the material can be isolated along a temperature gradient, as might be  
10 necessary if downstream uses require selecting, for example, the more fully hydroxylated materials, such as those sufficiently hydroxylated to retain triple helical structure at, e.g., body temperature (37°C).

In another embodiment, recombinant gelatins are produced in a system, e.g., a transgenic  
15 animal, in which hydroxylation is supplemented with exogenous prolyl hydroxylase. In one aspect, such a method of producing recombinant gelatins provides recombinant gelatins ranging from non-hydroxylated to fully-hydroxylated. The fraction of recombinant gelatins more fully hydroxylated will be substantially larger in recombinant material produced in the presence of exogenous prolyl hydroxylase than in recombinant material produced only in the  
20 presence of endogenous prolyl hydroxylase. Therefore, the melting temperatures of the material produced can range from, for example, 28°C to 40°C, having a median  $T_m$  value of around 34°C to 36°C. Such a gelatin mixture could be appropriate for use in a variety of applications, such as gel capsule manufacture, without requiring any fractionation or separation of differently hydroxylated portions.

25 The above methods provide for production of recombinant materials with a range of melting temperatures, that can be easily divided, for example, using a temperature gradient to separate materials solid at a particular temperature, e.g., 36°C, from those liquid at a particular temperature. Furthermore, the present invention provide for cost-effective methods of  
30 producing a material which, without separation, is suitable for use in bulk applications. For example, the manufacture of gel capsules could involve the use of recombinant gelatin produced by the above methods, wherein the recombinant material, having a range of melting temperatures, had a desirable melting temperature of around 33°C, such gelatin melting at body temperatures, and thus being suitable for swallowing and digestion. In the present  
35 methods, the recombinant gelatin can be produced directly in the desired system, e.g., a transgenic animal, or can be derived, for example, through hydrolysis, e.g., acid, thermal, or enzymatic, from recombinant collagens produced in the desired system.

5 In one embodiment, the present invention provides a method of producing recombinant gelatin comprising producing recombinant collagen and deriving recombinant gelatin from the recombinant collagen. In one aspect, the method comprises the expression of at least one polynucleotide sequence encoding a collagen or procollagen, or fragment or variant thereof, and at least one polynucleotide encoding a collagen post-translational enzyme or a subunit thereof. (See, e.g., U.S. Patent No. 5,593,859, incorporated by reference herein in its entirety.) The present recombinant gelatins can be derived from recombinant collagens using procedures known in the art. (See, e.g., Veis (1965) *Int Rev Connect Tissue Res*, 3:113-200.) For example, a common feature of all collagen-to-gelatin extraction processes is the loss of the secondary structure of the collagen protein, and in the majority of instances, an alteration in collagen structure. The collagens used in producing the gelatins of the present invention can be processed using different procedures depending on the type of gelatin desired.

Gelatin of the present invention can be derived from recombinantly produced collagen, or procollagens or other collagenous polypeptides, or from cell cultures, e.g., vertebrate cell cultures, by a variety of methods known in the art. For example, gelatin may be derived directly from the cell mass or the culture medium by taking advantage of gelatin's solubility at elevated temperatures and its stability under conditions of low or high pH, low or high salt concentrations, and high temperatures. Methods, processes, and techniques of producing gelatin compositions from collagen include digestion with proteolytic enzymes at elevated temperatures, denaturing the triple helical structure of the collagen utilizing detergents, heat, or various denaturing agents well known in the art, etc. In addition, various steps involved in the extraction of gelatin from animal or slaughterhouse sources, including treatment with lime or acids, heat extraction in aqueous solution, ion exchange chromatography, cross-flow filtration, and various methods of drying can be used to derive the gelatin of the present invention from recombinant collagen.

In one aspect, the gelatin of the present invention is comprised of denatured triple helices, and comprises at least one collagen subunit, collagen chain, or fragment thereof. The Gly-X-Y units within a particular collagen chain, subunit, or fragment thereof may be the same or different. Preferably, X and Y are either proline or hydroxyproline, and glycine appears in about every third residue position of the component chain. The amino acids of X and Y are proline or hydroxyproline, and each Gly-X-Y unit is the same or different. In another embodiment, the recombinant gelatin of the present invention comprises an amino acid sequence of  $(\text{Gly-X-Y})_n$ , wherein X and Y are any amino acid.

5

In one embodiment, the present gelatin is derived from a recombinant collagen of one type that is substantially free from collagen of any other collagen type. In one aspect, the recombinant collagen is type I collagen. In another aspect, the recombinant collagen is type III collagen. In another embodiment of the present invention, the recombinant collagen is human recombinant collagen. Further embodiments of the invention, in which the recombinant collagen is of any one collagen type, such as any one of collagen types I through XX, inclusively, or any other collagen, natural, synthetic, or semi-synthetic, are specifically contemplated. Embodiments in which the recombinant gelatin is derived from specified mixtures of any one or more of any of collagen types I through XX, inclusively, or any other collagen, natural, synthetic, or semi-synthetic, are specifically contemplated.

The present methods of producing recombinant gelatin have a number of advantages over traditional methods of gelatin extraction. Most importantly, the present methods provide a reliable non-tissue source of gelatin containing native collagen sequence. In addition, current methods of extraction do not allow for any natural source of human gelatin, such as might be advantageous for use in various medical applications. The present invention specifically provides recombinant gelatins derived from human sequences, compositions comprising recombinant human gelatins, and methods of producing these gelatins. The recombinant human gelatin is non-immunogenic as applied in pharmaceutical and medical processes, and various uses thereof are also contemplated.

In another aspect, the present invention provides for the production of the present gelatin from engineered constructs capable of expressing gelatin in various forms. This invention specifically contemplates methods of producing gelatin using recombinant prolyl hydroxylase and various synthetic constructs, including non-native collagen constructs. Further, the present invention provides recombinant gelatins that can be designed to possess the specific characteristics needed for a particular application. Methods for producing these gelatins are also contemplated. Using the current methods, one could produce a gelatin with the desired gel strength, viscosity, melting characteristics, isoelectric profile, pH, degree of hydroxylation, amino-acid composition, odor, color, etc. In one method according to the present invention, non-hydrolyzed gelatin is produced, and can be subsequently hydrolyzed fully or partially, if desired.

## 5    **Pr erties of Gelatin**

The various physical properties of gelatin define its usefulness in particular applications. Gelatin provides unique performance based on, for example, its amphoteric nature, its ability to form thermo-reversible gels, its protective colloidal and surface active properties, and its contribution to viscosity and stability. In a number of applications, gelatin is used, for  
10    example, as an emulsifier, thickener, or stabilizer; as an agent for film or coating formation; as a binding agent; as an adhesive or glue; or as a flocculating agent.

Raw materials, types of pre-treatment, and extraction processes all effect the composition of gelatin polypeptides obtained during conventional manufacture. Currently available animal  
15    products are thus heterogeneous protein mixtures of polypeptide chains. Gelatin molecules can be fairly large, with the molecular weight within a particular sample ranging from a few to several hundred kDa. The molecular weight distribution of gelatin in a particular lot can be critical, as weight distribution can influence, for example, the viscosity and/or gel strength of a gelatin sample.

20    In general, the viscosity of a gelatin solution increases with increasing concentration and with decreasing temperature. A higher viscosity solution would be preferred, for example, for gelatin used as a stabilizer or thickener. In some applications, liquid gelatins are preferred, such as in various emulsifying fluids, etc. Viscosity of a gelatin solution increases with  
25    increasing molecular weight of the gelatin components. A high-viscosity gelatin solution could consist, therefore, of a high concentration of low molecular weight gelatins, or of a lower concentration of high molecular weight gelatins. Viscosity also affects gel properties including setting and melting point. High-viscosity gelatin solutions provide gels with higher melting and setting rates than do lower viscosity gelatin solutions.

30    The thermoreversibility and thermoplasticity of gelatin are properties exploited in a number of applications, for example, in the manufacture of gel capsules and tablets. Gelatin can be heated, molded or shaped as appropriate, and cooled to form a capsule or tablet coating that has unique properties at homeostatic temperatures. The gelatin will begin to melt at mouth  
35    temperature, easing swallowing, and become liquid at body temperatures.

Gelatins of various gel strengths are suitable for use in different applications. The firmness or strength of the set gel is typically measured by calculating the Bloom value, which can be determined using international standards and methodology. Briefly, the Bloom strength is a

- 5 measurement of the strength of a gel formed by a 6.67% solution of gelatin in a constant temperature bath over 18 hours. A standard Texture Analyzer is used to measure the weight in grams required to depress a standard AOAC (Association of Official Agricultural Chemists) plunger 4 millimeters into the gel. If the weight in grams required for depression of the plunger is 200 grams, the particular gelatin has a Bloom value of 200. (See, e.g.,  
10 United States Pharmacopoeia and Official Methods of Analysis of AOAC International, 17<sup>th</sup> edition, Volume II.)

Commercial gelatins can thus be graded and sold on Bloom strength. Different ranges of Bloom values are appropriate for different uses of gelatin; for example, gelatins for use in  
15 various industrial applications, e.g., concrete stabilization, sand casting, molds, glues, coatings, etc., will be selected from a wide range of varying Bloom strengths, depending on the performance characteristics desired. Gelatins with varying Bloom strengths are also desired in the manufacture of various pharmaceutical products. For example, soft gel capsules are typically manufactured using ossein or skin gelatin with a Bloom value of about  
20 150 to 175 and/or porcine-derived gelatin with a Bloom value of about 190 to 210, or blends thereof, while hard gel capsules might use a gelatin with a Bloom value of about 220 to 260. In food applications, gelatin used, for example, as a thickener in marshmallows or other confectionary products might have a Bloom strength of around 250. Various applications, including certain emulsifying fluids in photographic applications, and various industrial  
25 coatings, involve the use of non-gelling gelatins.

The present invention provides for the production of recombinant gelatins with different Bloom strengths. In one aspect, the present invention provides, for example, for the manufacture of gelatins with Bloom strengths of around 50, 100, 150, 200, 250, and 300. In  
30 one embodiment, the present invention provides for the production of a recombinant gelatin having a Bloom strength of around 400. Such a gelatin can be used, for example, in the manufacture of gel capsules, and could allow for the manufacture of a lighter and thinner capsule, as less material would need to be used to provide a gel of sufficient strength. Recombinant gelatins with Bloom strengths of under 100, and from 0 to 100, inclusively, are  
35 also contemplated.

The present invention provides methods for designing recombinant gelatins with the physical properties desired for particular applications. In one embodiment, the present invention provides recombinant gelatins comprising uniform molecules of a specified molecular weight

5 or range of molecular weights, and methods for producing these recombinant gelatins. Such homogenous and uniform materials are advantageous in that they provide a reliable source of product with predictable performance, minimizing variability in product performance and in manufacturing parameters. Currently, gelatin from different lots must sometimes be blended in order to produce a mixture with the desired physical characteristics, such as the viscosity or  
10 gel strength, etc., provided by a particular molecular weight or molecular weight range.

In applications in which a specific molecular weight range of recombinant gelatin would be preferred to a recombinant gelatin with a specific molecular weight, the present invention provides such materials. Using the recombinant gelatins of the present invention, a  
15 manufacturer could, for example, mix recombinant gelatins from lots with specified molecular weights, in certain percentages, in order to achieve a mixture with the desired molecular weight range. Additionally, the present recombinant gelatins are inherently more uniform and of greater consistency than currently available commercial products. In one method of the present invention, recombinant collagen is processed, such as by acid or heat  
20 hydrolysis, to produce recombinant gelatin of a molecular weight range narrower than that of currently available gelatin products. Using suitable and controllable hydrolysis conditions, the present methods produced recombinant human gelatins with molecular weight distributions similar to those of commercially available gelatins, as well as recombinant gelatins with ranges narrower than those of the molecular weight ranges of currently available  
25 products. (See Examples 9 and 10.)

The present invention provides recombinant gelatins of uniform molecular weight or specified ranges of molecular weights, removing variability and unpredictability, and allowing for fine-tuning of processes and predictable behavior. The present methods allow for the production  
30 of recombinant gelatins of any desired molecular weight or range of molecular weights. For example, in one embodiment, the recombinant gelatin has a molecular weight greater than 300 kDa. In another embodiment, the recombinant gelatin has a molecular weight range of from about 150 to 250 kDa, or of from about 250 to 350 kDa. Other molecular weight ranges are specifically contemplated, including, but not limited to, the following molecular weight  
35 ranges: about 0 to 50 kDa, about 50 to 100 kDa, about 100 to 150 kDa, about 150 to 200 kDa, about 200 to 250 kDa, about 250 to 300 kDa, and about 300 to 350 kDa.

In another aspect, recombinant gelatin with a molecular weight similar to that of some commercially available gelatins, of from about 10 to 70 kDa, could be produced. In preferred



5     embodiments, the present invention provides recombinant gelatins narrower molecular weight ranges, not currently available in commercial products, such as from about 10 to 30 kDa, about 30 to 50 kDa, and about 50 to 70 kDa. In a particular embodiment, a recombinant gelatin with a chain length conferring specific properties appropriate to the intended application is provided. In various embodiments of the present invention, recombinant  
10    gelatins with uniform molecular weights of approximately 1 kDa, 5 kDa, 8 kDa, 9 kDa, 14 kDa, 16 kDa, 22 kDa, 23 kDa, and 44 kDa are contemplated. (See, e.g., Table 2.)

In particular, in one method of the present invention, gelatin is produced from shortened collagen sequences, for example, the sequences identified in Table 2. These sequences  
15    represent specific collagenous domains and encode short forms of gelatin.

The present gelatins are capable of retaining valuable physical characteristics of gelatin, for example, film-forming abilities, while possessing average molecular weights lower or higher than those of conventionally derived animal gelatin. Various modifications of collagen  
20    sequences, including, for example, denaturing of the collagen, collagen chain, subunit, or fragments thereof, or varying degrees of hydroxylation, can be made that will produce gelatin with specific physical properties, i.e., a higher or lower melting point than conventional gelatin, different amino acid compositions, specific molecular weights or ranges of molecular weights, etc., and such variations are specifically contemplated herein.

25    The molecular weight of a typical fibril-forming collagen molecule, such as type I collagen, is 300 kDa. In some applications, such as those in which high molecular weight gelatins are used, it might be desirable to produce a gelatin with a greater molecular weight than that of currently available extracted gelatin. Therefore, in one embodiment of the present invention,  
30    gelatin can be produced containing molecules larger than the collagen from which commercial gelatin is currently extracted. The resultant higher molecular weight gelatin product can be used directly in various applications in which its physical properties would be desirable, or can be divided and subsequently treated to produce molecules of a smaller sizes.

35    In one embodiment, gelatin can be produced using collagens larger than those available in conventional animal sources. For example, the present methods of production could be adapted to produce the acid-soluble cuticle collagens derived from the body walls of vestimentiferan tube worm *Riftia pachyptila* (molecular weight ~ 2600 kDa) and annelid *Alvinella pompejana* (molecular weight ~ 1700 kDa). These collagens could be adapted to

- 5 the present methods of production to produce larger molecules than those from which currently available gelatin is extracted, and the resultant product could be treated to produce gelatins as desired.

10 It is specifically contemplated that gelatins of various molecular weights can be produce by a variety of methods according to the present invention. For example, characteristics of the present recombinant gelatins, e.g., percentage hydroxylation, degrees of cross-linking, etc., can be varied to produce recombinant gelatins with the desired molecular weights. In one aspect, for example, the present invention provides a method for producing large molecular weight recombinant gelatins by using cross-linking agents known in the art to cross-link  
15 gelatin polypeptides. (See discussion, *infra*.)

In another aspect of the present invention, polypeptides from which gelatins could be derived are expressed from engineered constructs containing multiple copies of all or fragments of native collagen sequence. For example, in one embodiment, the present invention provides an  
20 altered collagen construct comprising multiple copies of the collagenous domain of type I collagen. In another embodiment, the construct comprises multiple copies of the collagenous domain of type III collagen. In a further embodiment, the construct comprises copies of type I and type III collagenous domains. The present invention provides for the use of single or multiple copies of all or portions of sequences encoding any collagen, including collagens  
25 type I through XX, inclusive. It is specifically contemplated that the present methods allow for the production of gelatins derived from more than one type of collagen. In one embodiment, recombinant gelatins derived from more than one type of collagen are co-expressed in an expression system, e.g., a host cell, transgenic animal, etc., such that a mixture of gelatins is produced.

30 In another embodiment, the present invention provides a method for producing gelatin without derivation from a collagen or procollagen triple helical stage. In one aspect, this involves production of recombinant gelatin by expression of various constructs in a high-temperature expression system, such as one relying on thermophilic organisms, that does not  
35 allow the formation of triple helical structures, but permits the activity of prolyl hydroxylase. The present gelatin could also be derived from collagen constructs containing mutations, additions, or deletions that prevent triple helical formation. In another aspect, this involves production of gelatin from shortened constructs that do not allow for formation of triple helices at regular temperatures, i.e., 37°C. Alternatively, gelatin can be produced in the

5 presence of inhibitors of triple helix formation, for example, polyanions, that are co-expressed with the biosynthetic collagen constructs. Additionally, the biosynthetic gelatin of the present invention could be derived from recombinantly produced collagen chains that do not form triple helices.

10 In another embodiment, the invention provides a method of deriving gelatin from non-hydroxylated collagen or collagen in which there is partial rather than full hydroxylation of proline residues. In one aspect, this method comprises deriving gelatin from collagen expressed in the absence of prolyl hydroxylase, for example, in an insect expression system without prolyl hydroxylase. (See, e.g., Myllyharju et al. (1997) J. Biol. Chem. 272, 21824-  
15 21830.) In one method according to the present invention, gelatin is derived from the partially hydroxylated or non-hydroxylated collagen. Hydroxylation can be conferred, for example, by *in vitro* administration of hydroxylases. In one method, a low degree of substitution of hydroxyproline for proline can be forced by providing hydroxyproline to, e.g., bacterial or yeast host cells.

20 The present invention comprises fully-hydroxylated, partially-hydroxylated, and non-hydroxylated recombinant gelatins. In another embodiment, the method of the present invention comprises producing a gelatin or gelatin precursor having a specific degree of hydroxylation. In a further aspect, the invention relates to a method of producing gelatin  
25 having from 20 to 80 percent hydroxylation, preferably, from about 30 to 60 percent hydroxylation, and, most preferably, about 40 percent hydroxylation. (See Examples 4 and 5.) The partially-hydroxylated recombinant gelatins of the present invention can be obtained through mixing specified percentages of recombinant gelatins with different degrees of hydroxylation, or can be obtained directly. (See Examples 4 and 5.) Further, the invention  
30 provides methods for achieving partial hydroxylation of recombinant gelatins by administering prolyl hydroxylase to non-hydroxylated recombinant gelatins *in vitro*, and controlling the length of the reaction.

There are limits to the extent to which the thermal characteristics of currently available  
35 animal-source gelatins can be altered. The present invention specifically provides for methods of producing recombinant gelatin, wherein the recombinant gelatin has the specific thermal characteristics desired for a particular application. Using the methods of the present invention, for example, the melting point and/or gel strength of the recombinant gelatin can be

5 manipulated in a variety of ways. The temperature stability and/or gel strength of recombinant gelatin can be measured by a variety of techniques well-known in the art.

Generally, the melting point of gelatin increases as the degree of hydroxylation increases. Using the methods of the present invention, it is possible to produce high molecular weight  
10 gelatins that, due to manipulation of hydroxylation and/or cross-linking, etc., have a lower gel strength and/or lower melting point than those of currently available animal-source gelatins. Therefore, the present invention provides a recombinant gelatin with properties unattainable in various commercial products, suitable for use in applications where a higher molecular weight gelatin is desired, in order to provide increased film strength, etc., but a non-gelling or  
15 low strength gel product is desired. In one embodiment, the present invention provides recombinant gelatin that has lower temperature stability due to incomplete hydroxylation of proline residues.

Such a recombinant gelatin could be useful in a variety of applications. In gelatin produced  
20 by current extraction methods, only fish gelatin provides a high average molecular weight film-forming protein that is non-gelling. The non-gelling and cold water-solubility characteristics offered by non-gelling fish gelatin can be matched by currently available hydrolyzed bovine and porcine gelatins, but with corresponding loss of film strength and flexibility, as the hydrolyzed gelatins are of lower average molecular weight. Therefore, in  
25 one embodiment, the present invention provides a partially-hydroxylated recombinant gelatin with lower gel strength and higher molecular weight than that provided by currently available animal-source materials.

A higher molecular weight, lower gel strength recombinant gelatin could also be useful in  
30 various pharmaceutical applications, in which stability is desired, but non- or low-gelling properties are desired in order to maintain the malleability and integrity of the pharmaceutical product. Such a recombinant gelatin could be used, for example, as a plasma expander, as its molecular weight could provide stability, increasing the residence time in circulation, and the altered setting point would prevent the material from gelling at room temperature, allowing  
35 the expander to be administered without warming. In one embodiment, the present invention provides a partially-hydroxylated recombinant gelatin suitable for use in pharmaceutical applications, for example, as a plasma expander.

- 5 In another aspect, partially-hydroxylated recombinant gelatin is obtained through expression of recombinant gelatin, or expression of polypeptides from which the present recombinant gelatin can be derived, in the absence of prolyl hydroxylase, for example, in an insect expression system without prolyl hydroxylase. (See, e.g., Myllyharju et al. (1997) J. Biol. Chem. 272, 21824-21830.) Hydroxylation can occur at the time of production or can be
- 10 subsequently imposed through, e.g., *in vitro* biological or chemical modification. In one method of the present invention, recombinant gelatins are derived from partially-hydroxylated or from fully hydroxylated collagen.

- Gelatins derived from natural sources by currently available methods are greatly strengthened
- 15 by the existence of covalent cross-links between lysine residues of the constituent collagen molecules. Cross-linking occurs naturally in the extracellular space following collagen secretion and fibril formation, as prior to secretion, certain lysine residues are hydroxylated by the enzyme lysyl hydroxylase. The extracellular enzyme lysyl oxidase subsequently deamidates certain lysine and hydroxylysine residues in the collagen molecules, yielding
- 20 highly reactive aldehyde groups that react spontaneously to form covalent bonds. The resulting cross-linked collagens yield gelatins of increased gel strength and increased viscosity. Specifically, a higher degree of cross-linking results in gelatins with higher melting temperatures and greater gel strength.

- 25 In one aspect, the present invention provides recombinant gelatins that are cross-linked, resulting in higher molecular weight gelatins. (See Example 7.) Cross-linking can be imposed by different methods, such as by biological or chemical modification. For example, in one embodiment, recombinant gelatin or a polypeptide from which gelatin can be derived is expressed in the presence of lysyl hydroxylase and lysyl oxidase. In another embodiment,
- 30 the polypeptide is modified by cross-linking after expression. In a further aspect, the present invention provides for imposition of cross-linking by chemical means, such as by reactive chemical cross-linkers, for example 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC). (See Example 7.) Other chemical cross-linking agents, such as bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>), 3,3'-dithiobis(sulfosuccinimidyl) propionate (DTSSP),
- 35 and Tris-sulfosuccinimidyl aminotriacetate (Sulfo-TSAT) may also be used, as can various agents known in the art. Additionally, the present invention provides methods of producing recombinant gelatins with varying degrees of cross-linking, useful for obtaining recombinant gelatins of desired melting points, gel strength, and viscosity.

- 5 The present invention provides methods to manipulate the molecular weight, the level of hydroxylation, and the degree of cross-linking of the recombinant gelatins to allow for creation of recombinant gelatins of different and specific Bloom strengths, as well as recombinant gelatins of different and specific levels of viscosity.
- 10 Proline hydroxylation plays central role in natural collagen formation. Hydroxylation of specific lysyl residues in the sequence X-Lys-Gly also performs an important function in collagen synthesis and fibril formation. The hydroxyl groups on modified lysine residues function as both attachment sites for carbohydrates and as essential sites for the formation of stable intermolecular cross-links. These modifications require the expression of specific
- 15 enzymes, lysyl hydroxylase and lysyl oxidase.

Therefore, in one aspect of the invention, the co-expression of these enzymes with the polypeptides of the present invention is contemplated. The gene encoding lysyl hydroxylase (Hautala et al. (1992) Genomics 13:62-69) is expressed in a host cell, which is then further

20 modified by the introduction of a sequence encoding a gelatin or polypeptide from which gelatin can be derived, as described in the present invention. The recombinant gelatins of the present invention can therefore be post-translationally modified by the activity of endogenously expressed lysyl hydroxylase and lysyl oxidase. The recombinant gelatins of the present invention can also be modified by the expression of exogenous lysyl hydroxylase and

25 lysyl oxidase. In one embodiment, recombinant gelatins produced are non-hydroxylated, and subsequently altered by imposing the desired degree of hydroxylation of lysine residues by the enzymatic activity of lysyl hydroxylase. The ability to alter the degree of lysyl hydroxylation is desirable in producing gelatins, and polypeptides from which gelatin can be derived, with various degrees of cross-linking that lead to the desired gel strengths and

30 viscosities.

In further embodiments, a polypeptide containing hydroxylysine residues can also be expressed in, for example, a yeast cell, in which hydroxyproline is produced by the activity of prolyl hydroxylase. (See Examples 1 and 4.) In some embodiments, the modified

35 recombinant gelatin or polypeptide from which gelatin can be derived can be formulated and administered to an animal or human, thus serving as a substrate for the activities of endogenous enzymes, such as lysyl oxidase, thus allowing the collagenous polypeptide to be incorporated into tissues in a stabilized cross-linked form. Therefore, one aspect of the present invention provides for the production of recombinant gelatins of desirable gel

- 5 strengths and viscosity for commercial use, without the need for lysyl hydroxylase or lysyl oxidase activities.

The invention also provides for the production of gelatin having a particular gelling point. In one embodiment, the present methods provide for the production of gelatin having a setting or  
10 gelling point of from 15 to 35°C. In further embodiments, the recombinant gelatin has a setting point of from 15 to 25°C, from 25 to 35°C, and from 20 to 30°C.

In various aspects, the present invention provides recombinant gelatin that is non-hydrolyzed, fully hydrolyzed, or hydrolyzed to varying degrees, such as gelatins that are a mixture of  
15 hydrolyzed and non-hydrolyzed products. Additionally, the present invention provides methods of producing recombinant gelatins with varying degrees of hydrolysis. (See Examples 9 and 10.) Gelatin hydrosylates are typically cold water-soluble and are used in a variety of applications, particularly in the pharmaceutical and food industries, in which a gelatin with non-gelling properties is desirable. Gelatin hydrolysates are used in the  
20 pharmaceutical industry in film-forming agents, micro-encapsulation processes, arthritis and joint relief formulas, tableting, and various nutritional formulas. In the cosmetics industry, gelatin hydrolysates are used in shampoos and conditioners, lotions and other formulations, including lipsticks, and in fingernail formulas, etc. Gelatin hydrolysates appear as nutritional supplements in protein and energy drinks and foods; are used as fining agents in wine, beer,  
25 and juice clarification; and are used in the micro-encapsulation of additives such as food flavorings and colors. Gelatin hydrosylates are used in industrial applications for their film-forming characteristics, such as in coatings of elements in semiconductor manufacture, etc.

In one embodiment of the present invention, gelatin is produced from collagen sequences in  
30 which particular native domains have been deleted or have been added in order to alter the behavior of the expressed product. The invention further contemplates methods of producing recombinant gelatin wherein the gelatin is produced directly from an altered collagen construct, without production of an intact triple helical collagen. In particular, the present invention contemplates methods of producing recombinant gelatin comprising the expression  
35 of various engineered constructs that do not encode standard triple helical collagen. For example, specific deletions can eliminate collagenase-responsive regions, and various regions eliciting immunogenic, e.g., antigenic and allergenic, responses.

5 Specific domains of various collagens have been associated with specific activities. (See, e.g., Shahan et al. (1999) *Con. Tiss. Res.* 40:221-232; Raff et al. (2000) *Human Genet* 106:19-28, both of which references are incorporated by reference herein in their entirety.) In particular, the present invention specifically provides for methods of producing recombinant gelatins derived from collagen constructs altered to eliminate or to reduce or  
10 increase specific regions of a collagen gene associated with a specific activity. Specifically, such regions could be deleted in full or in part to produce a gelatin lacking or with reduced specific activity, or additional copies of the specific region could be added to produce a gelatin with enhanced activity. For example, sequences in types I and III collagen recognized by the  $\alpha 2 \beta 1$  integrin receptor on the platelet cell surface have been identified. (Knight et al.  
15 (1998) *J. Biol. Chem.* 273:33287-33294; and Morton et al. (1997) *J. Biol. Chem.* 272:11044-11048, which references are incorporated by reference herein in their entirety.)

In one aspect of the present invention, it is desirable to create a homogenous gelatin composed of fragments synthesized from collagen constructs lacking platelet activation  
20 regions. Such gelatin could be included, for example, in products associated with anastomosis and vascular grafting, etc., including coatings for stent and graft devices. Such products can be associated with deleterious side effects, for example, thrombosis, that can develop in association with the use of such products as a result of the platelet-aggregating regions present in the collagenous product. In one aspect, the present invention provides for a  
25 method of producing a recombinant gelatin which can provide support for cell attachment when used in a stent or similar device, but which does not include platelet-reactive regions, thus minimizing the risk of platelet aggregation. (See Example 2.) Therefore, the present invention provides in one embodiment for a stent coating comprising recombinant gelatin. In a preferred embodiment, the recombinant gelatin is recombinant human gelatin. In some  
30 instances, such as various wound care applications, it could be desirable to provide recombinant gelatin comprising domains capable of inducing specific aggregating activities.

A gelatin of the present invention could be expressed from collagen constructs that did not encode the regions recognized by the  $\alpha 2 \beta 1$  receptor, or from constructs with one or with  
35 multiple copies of such regions, thus providing a homogenous and consistent gelatin product without or with reduced platelet aggregation and activation. In one aspect, the present invention provides for the production of recombinant gelatin, either through direct expression of gelatin or through processing of gelatin from collagenous polypeptides, through the use of highly efficient recombinant expression. The present production methods, as opposed to



5 current methods of extraction, offer extreme flexibility, as any one of a number of expression systems can be used. The production material is accessible, for example, in yeast or plant biomass. Secretion in certain production systems can be optimized, for example, by dictating the uniform size of particular gelatin molecules to be produced according to the present methods. In various embodiments, the present gelatins or the polypeptides from which these  
10 gelatins are derived, are produced in expression systems including, but not limited to, prokaryotic expression systems, such as bacterial expression systems, and eukaryotic expression systems, including yeast, animal, plant, and insect expression systems. Expression systems such as transgenic animals and transgenic plants are contemplated.

15 The present invention provides for expression of at least one polynucleotide encoding a gelatin or a polypeptide from which gelatin can be derived in a cell. In one embodiment, the present invention provides for the expression of more than one polynucleotide encoding a gelatin or a polypeptide from which gelatin can be derived in a cell, such that recombinant gelatin that is a homogenous or heterogeneous polypeptides is produced. The present  
20 invention further provides for expression of a polynucleotide encoding a collagen processing or post-translational enzyme or subunit thereof in a cell. Different post-translational modifications, and different post-translational enzymes, e.g., prolyl hydroxylase, lysyl hydroxylase, etc., can effect, for example, Bloom strength and other physical characteristics of the present gelatins.

25 The recombinant gelatins of the present invention are derived from collagenous sequences. The sequences from which the encoding polynucleotides of the invention are derived can be selected from human or from non-human sequences, depending on the characteristics desired for the intended use of the ultimate gelatin product. For pharmaceutical and medical uses,  
30 recombinant human gelatin is preferred. Non-human sources include non-human mammalian sources, such as bovine, porcine, and equine sources, and other animal sources, such as chicken and piscine sources. Non-native sequences are specifically contemplated.

Nucleic acid sequences encoding collagens have been generally described in the art. (See,  
35 e.g., Fuller and Boedtker (1981) *Biochemistry* 20:996-1006; Sandell et al. (1984) *J Biol Chem* 259:7826-34; Kohno et al. (1984) *J Biol Chem* 259:13668-13673; French et al. (1985) *Gene* 39:311-312; Metsaranta et al. (1991) *J Biol Chem* 266:16862-16869; Metsaranta et al. (1991) *Biochim Biophys Acta* 1089:241-243; Wood et al. (1987) *Gene* 61:225-230; Glumoff et al. (1994) *Biochim Biophys Acta* 1217:41-48; Shirai et al. (1998) *Matrix Biology* 17:85-88;

- 5 Tromp et al. (1988) Biochem J 253:919-912; Kuivaniemi et al. (1988) Biochem J 252:633-640; and Ala-Kokko et al. (1989) Biochem J 260:509-516.) See also co-pending, commonly-owned application U.S. Application Serial No. \_\_\_\_\_, entitled "Animal Collagens and Gelatins," filed 10 November 00, incorporated herein by reference in its entirety.)
- 10 The nucleic acid sequences of the invention may be engineered in order to alter the coding sequences used to produce recombinant gelatin, or polypeptides from which the recombinant gelatin can be derived, for a variety of ends including, but not limited to, alterations which modify processing and expression of the gene product. For example, alternative secretory
- 15 signals may be substituted for any native secretory signals. Mutations may be introduced using techniques well known in the art, e.g., site-directed mutagenesis, PCR-directed mutagenesis, cassette mutagenesis, and other techniques well-known in the art to insert new restriction sites, or to alter glycosylation patterns, phosphorylation, proteolytic turnover/breakdown, etc. Additionally, when producing gelatin in an expression system using particular host cells, the polynucleotides of the invention may be modified in the silent position of any triplet amino acid
- 20 codon so as to better conform to the codon preference of a particular host organism.

Altered polynucleotide sequences which may be used in accordance with the invention include sequences containing deletions, additions, or substitutions of nucleotide residues in native collagen sequences. Such polynucleotides can encode the same or a functionally equivalent

25 gene product. The gene product itself may contain deletions, additions or substitutions of amino acid residues within a collagen sequence.

The polynucleotide sequences of the invention are further directed to sequences which encode variants of the encoded polypeptides. The encoded amino acid variants may be prepared by

30 various methods known in the art for introducing appropriate nucleotide changes for encoding variant polypeptides. Two important variables in the construction of amino acid sequence variants are the location of the mutation and the nature of the mutation. The amino acid sequence variants of the gelatins of the present invention, or of the polypeptides from which the present gelatins are derived, are preferably constructed by mutating the polynucleotide to give an

35 amino acid sequence that does not occur in nature. These amino acid alterations can be made at sites that differ in, for example, collagens from different species (variable positions), or in highly conserved regions (constant regions). Sites at such locations will typically be modified in series, e.g., by substituting first with conservative choices (e.g., hydrophobic amino acid to a different

- 5 hydrophobic amino acid) and then with more distant choices (e.g., hydrophobic amino acid to a charged amino acid), and then deletions or insertions may be made at the target site.

Due to the inherent degeneracy of the genetic code, other nucleic acid sequences which encode substantially the same or a functionally equivalent amino acid sequence or  
10 polypeptide, natural, synthetic, semi-synthetic, or recombinant in origin, may be used in the practice of the claimed invention. Degenerate variants are specifically contemplated by the present invention, including codon-optimized sequences. In addition, the present invention specifically provides for polynucleotides which are capable of hybridizing to a particular sequence under stringent conditions.

15

#### Expression

The present methods are suitably applied to the range of expression systems available to those of skill in the art. While a number of these expression systems are described below, it is to be understood that application of the present methods not limited to the specific embodiments set  
20 forth below.

A variety of expression systems may be utilized to contain and express sequences encoding the recombinant gelatins of the present inventions or encoding polypeptides from which these gelatins can be derived. These include, but are not limited to, microorganisms such as bacteria  
25 transformed with recombinant bacteriophage, plasmid, or cosmid nucleic acid expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g., baculovirus); filamentous fungi transformed with fungal vectors; plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., pET or  
30 pBR322 plasmids); or animal cell systems.

Control elements or regulatory sequences suitable for use in expressing the polynucleotides of the present invention are those non-translated regions of the vector, including enhancers, promoters, and 5' and 3' untranslated regions, which interact with host cellular proteins to  
35 carry out transcription and translation. Such elements may vary in strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements may be used.

5 Promoters are untranslated sequences located upstream from the start codon of the structural gene that control the transcription of the nucleic acid under its control. Inducible promoters are promoters that alter their level of transcription initiation in response to a change in culture conditions, e.g., the presence or absence of a nutrient. One of skill in the art would know of a large number of promoters that would be recognized in host cells suitable for use in the methods  
10 of the present invention.

Promoter, enhancer, and other control elements can be selected as suitable by one skilled in the art. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid (Stratagene, La Jolla, Calif.) or pSPORT1  
15 plasmid (GIBCO BRL) and the like may be used. In insect cells, the baculovirus polyhedrin promoter may be used. In plant systems, promoters or enhancers derived from the genomes of plant cells (e.g., heat shock promoter, the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein; promoters for various storage protein genes, etc.) or from plant viruses (e.g., viral promoters or leader sequences, the 35S RNA promoter of  
20 CaMV, the coat protein promoter of TMV, etc.) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes (e.g., metallothionein promoter, -actin promoter, etc.) or from mammalian viruses (e.g., the adenovirus late promoter, CMV, SV40, LTR, TK, and the vaccinia virus 7.5 K promoters, etc.) are preferable. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding the desired  
25 polypeptide, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

Such promoters can be operably linked to the polynucleotides encoding the gelatin or gelatin precursors of the present invention, such as by removing the promoter from its native gene and placing the encoding polynucleotide at the 3' end of the promoter sequence. Promoters useful in  
30 the present invention include, but are not limited to, prokaryotic promoters, including, for example, the lactose promoter, arabinose promoter, alkaline phosphatase promoter, tryptophan promoter, and hybrid promoters such as the tac promoter; yeast promoters, including, for example, the promoter for 3-phosphoglycerate kinase, other glycolytic enzyme promoters (hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase,  
35 etc.), the promoter for alcohol dehydrogenase, the alcohol oxidase (AOX) 1 or 2 promoters, the metallothionein promoter, the maltose promoter, and the galactose promoter; and eukaryotic promoters, including, for example, promoters from the viruses polyoma, fowlpox, adenovirus, bovine papilloma virus, avian sarcoma virus, cytomegalovirus, retroviruses, SV40, and promoters from the target eukaryote, for example, the glucoamylase promoter from *Aspergillus*,

- 5 actin or ubiquitin promoters, an immunoglobulin promoter from a mammal, and native collagen promoters. (See, e.g., de Boer et al. (1983) *Proc. Natl. Acad. Sci. USA* 80:21-25 ; Hitzeman et al. (1980) *J. Biol. Chem.* 255:2073); Fiers et al. (1978) *Nature* 273:113; Mulligan and Berg (1980) *Science* 209:1422-1427; Pavlakis et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:7398-7402; Greenway et al. (1982) *Gene* 18:355-360; Gray et al. (1982) *Nature* 295:503-508; Reyes et al. (1982) *Nature* 297:598-601; Canaani and Berg (1982) *Proc. Natl. Acad. Sci. USA* 79:5166-5170; Gorman et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:6777-6781; and Nunberg et al. (1984) *Mol. and Cell. Biol.* 11(4):2306-2315.)

- 15 The polynucleotide sequences encoding the gelatins and gelatin precursors of the present methods may be under the transcriptional control of a constitutive promoter, directing expression generally. Alternatively, the polynucleotides employed in the present methods are expressed in a specific tissue or cell type, or under more precise environmental conditions or developmental controls. Promoters directing expression in these instances are known as inducible promoters. In the case where a tissue-specific promoter is used, protein expression
- 20 is particularly high in the tissue from which extraction of the protein is desired. In plants, for example, depending on the desired tissue, expression may be targeted to the endosperm, aleurone layer, embryo (or its parts as scutellum and cotyledons), pericarp, stem, leaves, tubers, roots, etc. Examples of known tissue-specific promoters in plants include the tuber-directed class I patatin promoter, the promoters associated with potato tuber ADPGPP genes, the soybean promoter of  $\beta$ -conglycinin (7S protein), which drives seed-directed transcription, and seed-directed promoters from the zein genes of maize endosperm. (See, e.g., Bevan et al. (1986) *Nucleic Acids Res.* 14: 4625-4638; Muller et al. (1990) *Mol. Gen. Genet.* 224: 136-146; Bray (1987) *Planta* 172:364-370 ; and Pedersen et al. (1982) *Cell* 29:1015-1026.)
- 30 Transcription of the sequences encoding the gelatins or gelatin precursors of the present invention from the promoter is often increased by inserting an enhancer sequence in the vector. Enhancers are cis-acting elements, usually about from 10 to 300 bp, that act to increase the rate of transcription initiation at a promoter. Many enhancers are known for both eukaryotes and prokaryotes, and one of ordinary skill could select an appropriate enhancer for the host cell of
- 35 interest. (See, e.g., Yaniv (1982) *Nature* 297:17-18.)

The gelatins and gelatin precursors of the present invention may be expressed as secreted proteins. When the engineered cells used for expression of the proteins are non-human host cells, it is often advantageous to replace the secretory signal peptide of the collagen protein with

5 an alternative secretory signal peptide which is more efficiently recognized by the host cell's secretory targeting machinery. The appropriate secretory signal sequence is particularly important in obtaining optimal fungal expression of mammalian genes. (See, e.g., Brake et al. (1984) Proc. Natl. Acad. Sci. USA 81:4642.) Other signal sequences for prokaryotic, yeast, fungi, insect or mammalian cells are well known in the art, and one of ordinary skill could easily  
10 select a signal sequence appropriate for the host cell of choice.

The efficiency of expression may be enhanced by the inclusion of enhancers appropriate for the particular cell system which is used, such as those described in the literature. (See, e.g., Scharf, D. et al. (1994) Results Probl. Cell Differ. 20:125-162.) In addition, a host cell strain may be  
15 chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, prenylation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding, and/or function. Different host cells such as  
20 CHO, HeLa, MDCK, HEK293, and WI38, which have specific cellular machinery and characteristic mechanisms for such post-translational activities, may be chosen to ensure the correct modification and processing of the foreign protein.

In accordance with the invention, polynucleotide sequences encoding recombinant gelatins or  
25 polypeptides from which gelatins can be derived may be expressed in appropriate host cells. In preferred embodiments of the invention, the recombinant gelatin is human gelatin. In other preferred embodiments of the invention, the polynucleotide sequences are derived from type I collagen sequence, free of coding sequence for any other type of collagen, or from type II collagen, free of coding sequence for any other type of collagen, or from type III collagen, free  
30 of coding sequence for any other type of collagen. In another embodiment, the encoding polynucleotides are derived from type I and type III collagen in specified quantities, such that the gelatin produced by or derived from the encoded polypeptides comprises a mixture of type I and type III collagens in defined quantities.

35 In order to express the collagens from which the present gelatins are derived, or to express sequences other than natural collagen sequences leading to the production of the present gelatin, nucleotide sequences encoding the collagen, or a functional equivalent, or other sequence, for example, a shortened collagen sequence, such as those presented in Table 2, is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the

- 5 transcription and translation of the inserted coding sequence, or in the case of an RNA viral vector, the necessary elements for replication and translation.

Methods well-known to those skilled in the art can be used to construct expression vectors containing the desired coding sequence and appropriate transcriptional/translational control  
10 signals. These methods include standard DNA cloning techniques; e.g., *in vitro* recombinant techniques, synthetic techniques, and *in vivo* recombination. See, for example, the techniques described in Maniatis et al., *supra*; Ausubel et al., *supra*; and Ausubel, F.M. (1997) Short Protocols in Molecular Biology, John Wiley and Sons, New York, NY.

- 15 Various expression vectors may be used to express the present polypeptides. For example, a typical expression vector contains elements coding for a replication origin; a cloning site for insertion of an exogenous nucleotide sequence; elements that control initiation of transcription of the exogenous gene, such as a promoter; and elements that control the processing of transcripts, such as a transcription/termination/polyadenylation sequence. An  
20 expression vector for use in the present invention can also contain such sequences as are needed for the eventual integration of the vector into the chromosome. In addition, a gene that codes for a selection marker which is functionally linked to promoters that control transcription initiation may also be within the expression vector, for example, an antibiotic resistance gene to provide for the growth and selection of the expression vector in the host.

- 25 The vectors of this invention may autonomously replicate in the host cell, or may integrate into the host chromosome. Suitable vectors with autonomously replicating sequences are well known for a variety of bacteria, yeast, and various viral replications sequences for both prokaryotes and eukaryotes. Vectors may integrate into the host cell genome when they have a  
30 DNA sequence that is homologous to a sequence found in host cell genomic DNA.

- For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the present polypeptides may be transformed using expression vectors containing viral origins of replication or appropriate expression  
35 elements (e.g., promoters, enhancers, transcription terminators, polyadenylation sites, etc.) and a selectable marker gene on the same or on a separate vector. Following the introduction of the vectors, cells may be allowed to grow for 1-2 days in enriched media, and are then switched to selective media. The selectable marker in the recombinant plasmid confers resistance to selection, allowing growth and recovery of cells that successfully express the

5 introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type. This method may advantageously be used to produce cell lines which express a desired polypeptide.

Expression of the various sequences used in the methods of the present invention driven by,  
10 for example, the galactose promoters can be induced by growing the culture on a non-repressing, non-inducing sugar so that very rapid induction follows addition of galactose; by growing the culture in glucose medium and then removing the glucose by centrifugation and washing the cells before resuspension in galactose medium; and by growing the cells in  
15 medium containing both glucose and galactose so that the glucose is preferentially metabolized before galactose-induction can occur.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase and adenine phosphoribosyl-transferase genes which can be employed in *tk* or *aprt* cells, respectively.  
20 (See, e.g., Wigler, M. et al. (1977) Cell 11:223-32; Lowy, I. et al. (1980) Cell 22:817-23.) Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection. Therefore, the present invention contemplates the use of such selectable markers, for example: *dhfr*, which confers resistance to methotrexate; *npt*, which confers resistance to the aminoglycosides neomycin and G-418; and *als* or *pat*, which confer resistance to  
25 chlorsulfuron and to phosphinotricin acetyltransferase, respectively. (See, e.g., Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. 77:3567-3570; and Colbere-Garapin, F. et al. (1981) J. Mol. Biol. 150:1-14.)

Additional selectable genes have been described, for example, *trpB*, which allows cells to utilize  
30 indole in place of tryptophan, or *hisD*, which allows cells to utilize histinol in place of histidine. (See, e.g., Hartman, S. C. and R. C. Mulligan (1988) Proc. Natl. Acad. Sci. 85:8047-51.) Recently, the use of visible markers has gained popularity with such markers as anthocyanins,  $\beta$ -glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, now widely used  
35 not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system. (See, e.g., Rhodes, C. A. et al. (1995) Methods Mol. Biol. 55:121-131.)

As noted above, the expression vectors for use in the present methods of production can typically comprise a marker gene that confers a selectable phenotype on cells. Usually, the



5 selectable marker gene will encode antibiotic resistance, with suitable genes including at least one set of genes coding for resistance to the antibiotic spectinomycin, the streptomycin  
phosphotransferase (SPT) gene coding for streptomycin resistance, the neomycin  
phosphotransferase (NPTII) gene encoding kanamycin or geneticin resistance, the hygromycin  
10 resistance gene, genes coding for resistance to herbicides which act to inhibit the action of acetolactate synthase (ALS), in particular, the sulfonylurea-type herbicides (e.g., the S4 and/or  
Hra mutations), genes coding for resistance to herbicides which act to inhibit action of glutamine  
synthase, such as phosphinothricin or basta (e.g. the bar gene), or other similar genes known in  
the art. The bar gene encodes resistance to the herbicide basta, the *hptII* gene encodes resistance  
to the antibiotics kanamycin and geneticin, and the ALS gene encodes resistance to the herbicide  
15 chlorsulfuron.

Other methods for determining which host cells, subsequent to transformation, contain the  
polynucleotides of interest include a variety of procedures known to those of skill in the art.  
These procedures include, but are not limited to, nucleic acid hybridizations, including DNA-  
20 DNA or DNA-RNA hybridizations, and various protein bioassay or immunoassay techniques  
including membrane-, solution-, or chip-based technologies for the detection and/or  
quantification of polynucleotides or polypeptides.

In addition, a host cell strain may be chosen which modulates the expression of the inserted  
25 sequences, or modifies and processes the gene product in the specific fashion desired. Such  
modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be  
important for the function of the protein. Different host cells have characteristic and specific  
mechanisms for the post-translational processing and modification of proteins. Appropriate cell  
lines or host systems can be chosen to ensure the correct modification and processing of the  
30 foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery  
for proper processing of the primary transcript, including various modifications such as protein  
folding, disulfide bond formation, glycosylation, and phosphorylation of the gene product may  
be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa,  
COS, MDCK, 293, WI38, etc.

35 Specific initiation signals may also be used to achieve more efficient translation of the  
polynucleotides of the present invention. Such signals include the ATG initiation codon and  
adjacent sequences. In cases where sequences encoding the present polypeptides, along with  
any initiation or upstream sequences required for translation, etc., are inserted into the

5 appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequences, or portions thereof, are inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be  
10 of various origins, both natural and synthetic. (See, e.g., Bittner et al. (1987) Meth. in Enzymol. 153:516-544.)

A host cell of the present invention can be infected, transfected, or transformed with at least one polynucleotide encoding a post-translational enzyme, in addition to at least one polynucleotide  
15 encoding a gelatin of the present invention or a polypeptide from which the gelatin can be derived. Such polynucleotides include those encoding collagen post-translational enzymes, such as prolyl 4-hydroxylase, collagen glycosyl transferase, C-proteinase, N-proteinase, lysyl oxidase, or lysyl hydroxylase, and can be inserted into cells that do not naturally produce post-translational enzymes, for example, into yeast cells, or cells that may not naturally produce  
20 sufficient amounts of post-translational enzymes, for example, various insect and mammalian cells, such that exogenous enzyme may be required to produce certain post-translational effects. In one embodiment of the present invention, the post-translational enzyme is prolyl 4-hydroxylase, and the polynucleotide encodes the  $\alpha$  or the  $\beta$  subunit of prolyl hydroxylase. In a preferred embodiment, polynucleotides encoding the  $\alpha$  subunit and the  $\beta$  subunit of prolyl 4-  
25 hydroxylase are inserted into a cell to produce a biologically active prolyl 4-hydroxylase enzyme, co-expressed with a polynucleotide encoding a gelatin or a polypeptide from which gelatin can be derived.

The polynucleotides encoding post-translational enzymes may be derived from any source,  
30 whether natural, synthetic, or recombinant. In a preferred embodiment, the post-translational enzyme is derived from the same species as is the recombinant gelatin to be produced. In one embodiment, the recombinant gelatin to be produced is human recombinant gelatin, and the post translational enzyme is human prolyl 4-hydroxylase.

35 The expressed gelatins or gelatin precursors of the present invention are preferably secreted into culture media and can be purified to homogeneity by methods known in the art, for example, by chromatography. In one embodiment, the recombinant gelatin or gelatin precursors are purified by size exclusion chromatography. However, other purification techniques known in the art can also be used, including, but not limited to, ion exchange chromatography, hydrophobic

- 5 interaction chromatography (HIC), and reverse-phase chromatography. (See, e.g., Maniatis et al., *supra*; Ausubel et al., *supra*; and Scopes (1994) Protein Purification: Principles and Practice, Springer-Verlag New York, Inc., NY.)

#### Prokaryotic

- 10 In prokaryotic systems, such as bacterial systems, any one of a number of expression vectors may be selected, depending upon the use intended for the polypeptides to be expressed. For example, when large quantities of the recombinant gelatins of the present invention, or polypeptides from which these recombinant gelatins can be derived, are needed, vectors which direct high-level expression of fusion proteins that can be readily purified may be used.
- 15 Such vectors include, but are not limited to, multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene), in which the encoding sequence may be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent seven residues of  $\beta$ -galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke, G. and S. M. Schuster (1989) *J. Biol. Chem.* 264:5503-5509); and the like. pGEX (Promega,
- 20 Madison, Wis.) and pET (Invitrogen) vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by a variety of methods known in the art, for example, by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems may be designed to include
- 25 heparin, thrombin, or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

#### Yeast

- In preferred embodiments, the present invention provides methods of producing recombinant
- 30 gelatin using a yeast expression system. In preferred embodiments, gelatin is produced directly from altered collagen constructs or derived from processing of collagenous polypeptides. A number of vectors containing constitutive, non-constitutive, or inducible promoters may be used in yeast systems. (See, e.g., Ausubel et al., *supra*, Chapter 13.) In some aspects, vectors containing sequences which direct DNA integration into the chromosome are used for
- 35 expression in *S. cerevisiae*.

In one embodiment, the recombinant gelatins of the invention, or the polypeptides from which these gelatins can be derived, are expressed using host cells from the yeast *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* can be used with any of a large number of expression

5 vectors available in the art, including a number of vectors containing constitutive or inducible promoters such as  $\alpha$  factor, AOX, GAL1-10, and PGH. (See, e.g., Ausubel et al., *supra*, and Grant et al. (1987) *Methods Enzymol.* 153:516-544.) Commonly employed expression vectors are shuttle vectors containing the  $2\mu$  origin of replication for propagation both in yeast and the ColE1 origin for *E. coli*, including a yeast promoter and terminator for efficient transcription of  
10 the foreign gene. Vectors incorporating  $2\mu$  plasmids include, but are not limited to, pWYG4 and pYES2, which have the  $2\mu$  ORI-STB elements, the GAL1-10, etc. In one method of the present invention, in which a hydroxylated product is desired, involves the co-expression of a collagen post-translational enzyme, for example, prolyl 4-hydroxylase. In one such method, using the pWYG4 vector, the NcoI cloning site is used to insert the gene for either the  $\alpha$  or  $\beta$  subunit of  
15 prolyl 4-hydroxylase, and to provide the ATG start codon for either the  $\alpha$  or  $\beta$  subunit. In one method, expression plasmids are used which direct integration into the chromosome of the host.

The expression vector pWYG7L, which has intact  $2\alpha$  ORI, STB, REP1 and REP2, the GAL7 promoter, and the FLP terminator, can also be used. When the co-expression of a post-  
20 translational enzyme, for example, prolyl 4-hydroxylase, is desired, the gene for either the  $\alpha$  or  $\beta$  subunit of prolyl 4-hydroxylase is inserted in the polylinker with its 5' ends at a BamHI or NcoI site. The vector containing the prolyl 4-hydroxylase gene is transformed into *S. cerevisiae* either before or after removal of the cell wall to produce spheroplasts that take up DNA on treatment with calcium and polyethylene glycol or by treatment of intact cells with lithium ions.  
25 Alternatively, DNA can be introduced by electroporation. Transformants can be selected by using host yeast cells that are auxotrophic for leucine, tryptophane, uracil or histidine together with selectable marker genes such as LEU2, TRP1, URA3, HIS3 or LEU2-D.

In another preferred embodiment, the methods of producing recombinant gelatin according to  
30 the present invention use host cells from the yeast *Pichia pastoris*, or from other species of non-*Saccharomyces* yeast, that possess advantages in producing high yields of recombinant protein in scaled-up procedures. *Pichia* expression systems include advantages of both prokaryotic (e.g., *E. coli*) expression systems – high-level expression, easy scale-up, and inexpensive growth – and eukaryotic expression systems – protein processing, folding, and  
35 post-translational modifications. Such expression systems can be constructed using various methods and kits available to those skilled in the art, for example, the PICHIA EXPRESSION kits available from Invitrogen Corporation (San Diego, CA).

5 There are a number of methanol responsive genes in methylotrophic yeasts such as *Pichia*  
*pastoris*, or *Pichia methanolica*, etc., the expression of each being controlled by methanol  
responsive regulatory regions (also referred to as promoters). Any of such methanol  
responsive promoters are suitable for use in the practice of the present invention. Examples  
of specific regulatory regions include the promoter for the primary alcohol oxidase gene from  
10 *Pichia pastoris* AOX1, the promoter for the secondary alcohol oxidase gene from *Pichia*  
*pastoris* AOX2, the FLD1 promoter, the promoter for the dihydroxyacetone synthase gene  
from *Pichia pastoris* (DAS), the promoter for the P40 gene, etc. Typically, expression in  
*Pichia pastoris* is obtained by the promoter from the tightly regulated AOX1 gene. (See, e.g.,  
Ellis et al. (1985) Mol. Cell. Biol. 5:1111; and U.S. Patent No. 4,855,231.) Constitutive  
15 expression can also be achieved using, e.g., the GPH promoter.

Another yeast expression system preferred for use in the methods of the present invention makes  
use of the methylotrophic yeast *Hansenula polymorpha*. This system can be used, for example,  
in a method of production of the present invention where high yield is desirable. Growth on  
20 methanol results in the induction of enzymes key in, such as MOX (methanol oxidase), DAS  
(dihydroxyacetone synthase), and FMHD (formate dehydrogenase). These enzymes can  
constitute up to 30-40% of the total cell protein. The genes encoding MOX, DAS, and FMDH  
production are controlled by strong promoters induced by growth on methanol and repressed by  
growth on glucose. Any or all three of these promoters may be used to obtain high level  
25 expression of heterologous sequences in *H. polymorpha*, according to methods known in the art.

In one method of the present invention, the encoding polynucleotides are cloned into an  
expression vector under the control of an inducible *H. polymorpha* promoter. If secretion of the  
product is desired, a polynucleotide encoding a signal sequence for secretion in yeast, such as  
30 MF $\alpha$ 1, is fused in frame with the coding sequence for the polypeptides of the invention. The  
expression vector preferably contains an auxotrophic marker gene, such as URA3 or LEU2, or  
any other marker known in the art, which may be used to complement the deficiency of an  
auxotrophic host. Alternatively, dominant selectable markers such as zeocin or blastacin may be  
used.

35 The expression vector is then used to transform *H. polymorpha* host cells using techniques  
known to those of skill in the art. An interesting and useful feature of *H. polymorpha*  
transformation is the spontaneous integration of up to 100 copies of the expression vector into  
the genome. In most cases, the integrated sequences form multimers exhibiting a head-to-tail

- 5 arrangement. The integrated foreign DNA has been shown to be mitotically stable in several recombinant strains, even under non-selective conditions. This phenomenon of high copy integration further adds to the productivity potential of the system.

#### Plant

- 10 The present invention also contemplates the production of the recombinant gelatin of the present invention, or polypeptides from which the recombinant gelatin can be derived, in plant expression systems, including plant host cells and transgenic plants. (See, e.g., Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins, Owen and Pen, eds., John Wiley & Sons, 1996; Transgenic Plants, Galun and Breiman, eds.,  
15 Imperial College Press, 1997; and Applied Plant Biotechnology, Chopra et al. eds., Science Publishers, Inc., 1999.) In cases where plant expression vectors are used, the expression of sequences may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV. (See, e.g., Brisson et al. (1984) Nature 310:511-514; and  
20 Takamatsu, N. (1987) EMBO J. 6:307-311.) Plant expression vectors and reporter genes are generally known in the art. (See, e.g., Gruber et al. (1993) in Methods of Plant Molecular Biology and Biotechnology, CRC Press.)

- Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock  
25 promoters e.g., soybean hsp17.5-E or hsp17.3-B may be used. (See, e.g., Coruzzi, G. et al. (1984) EMBO J. 3:1671-1680; Broglie, R. et al. (1984) Science 224:838-843; Winter, J. et al. (1991) Results Probl. Cell Differ. 17:85-105; and Gurley et al. (1986) Mol. Cell. Biol. 6:559-565.) These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, pathogen-mediated transfection, particle bombardment, or any other means known in the art, such as are  
30 described in a number of generally available reviews. (See, e.g., Hobbs, S. or Murry, L. E. in McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York, N.Y., pp. 191-196; Weissbach and Weissbach (1988) Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson and Corey, Plant Molecular  
35 Biology, 2d Ed., Blackie, London, Ch. 7-9.)

In various embodiments, the recombinant gelatin of the present invention, or polypeptides from which the present recombinant gelatin can be derived, is produced from seed by way of available seed-based production techniques using, for example, canola, corn, soybeans, rice,

- 5 and barley seed. In such embodiments, the protein is recovered during seed germination/molting. In other embodiments, the protein is expressed directly into the endosperm or into other parts of the plant so that the gelatin is non-extracted, and the plant itself can serve as, for example, a dietary supplement such as a source of protein.
- 10 Promoters that may be used to direct the expression of the polynucleotides may be heterologous or non-heterologous. These promoters can also be used to drive expression of antisense nucleic acids to reduce, increase, or alter expression as desired. Other modifications may be made to increase and/or maximize transcription of sequences in a plant or plant cell are standard and known to those in the art. For example, the polynucleotide sequences
- 15 operably linked to a promoter may further comprise at least one factor that modifies the transcription rate of the encoded polypeptides, such as, for example, peptide export signal sequence, codon usage, introns, polyadenylation signals, and transcription termination sites. Methods of modifying nucleic acid constructs to increase expression levels in plants are generally known in the art. (See, e.g. Rogers et al. (1985) J. Biol. Chem. 260:3731; Comejo
- 20 et al. (1993) Plant Mol Biol 23:567-568.) In engineering a plant system that affects the rate of transcription of the polynucleotides, various factors known in the art, including regulatory sequences such as positively or negatively acting sequences, enhancers and silencers, chromatin structure, etc., can be used.
- 25 Typical vectors useful for expression of foreign genes in plants are well known in the art, including, but not limited to, vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens*. These vectors are plant integrating vectors, that upon transformation, integrate a portion of the DNA into the genome of the host plant. (See, e.g., Rogers et al. (1987) Meth. In Enzymol. 153:253-277; Schardl et al. (1987) Gene 61:1-11; and
- 30 Berger et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:8402-8406.)

Procedures for transforming plant cells are available in the art, including, for example, direct gene transfer, *in vitro* protoplast transformation, plant virus-mediated transformation, liposome-mediated transformation, microinjection, electroporation, *Agrobacterium*-mediated

35 transformation, and ballistic particle acceleration. (See, e.g., Paszkowski et al. (1984) EMBO J. 3:2717-2722; U.S. Patent No. 4,684,611; European Application No. 0 67 553; U.S. Patent No. 4,407,956; U.S. Patent No. 4,536,475; Crossway et al. (1986) Biotechniques 4:320-334; Riggs et al. (1986) Proc. Natl. Acad. Sci USA 83:5602-5606; Hinchey et al. (1988) Biotechnology 6:915-921; and U.S. Patent No. 4,945,050.) Standard methods for the

- 5 transformation of rice, wheat, corn, sorghum, and barley are described in the art. (See, e.g., Christou et al. (1992) Trends in Biotechnology 10:239; Casas et al. (1993) Proc. Nat'l Acad. Sci. USA 90:11212; Wan et al. (1994) Plant Physiol. 104:37; and Lee et al. (1991) Proc. Nat'l Acad. Sci. USA 88: 6389.) Wheat can be transformed by techniques similar to those employed for transforming corn or rice. (See, e.g., Fromm et al. (1990) Bio/Technology 8:833; and Gordon-Kamm et al., *supra*.)
- 10

Additional methods that may be used to generate plants or plant cells that can express the present recombinant gelatins, or polypeptides from which these recombinant gelatins can be derived, are well-established in the art. (See, e.g., U.S. Patent No. 5,959,091; U.S. Patent No. 5,859,347; U.S. Patent No. 5,763,241; U.S. Patent No. 5,659,122; U.S. Patent No. 5,593,874; U.S. Patent No. 5,495,071; U.S. Patent No. 5,424,412; U.S. Patent No. 5,362,865; and U.S. Patent No. 5,229,112.)

15

The present invention further provides a method of producing polypeptides by providing a biomass from plants or plant cells which are comprised of at least one polynucleotide sequence encoding a recombinant gelatin, or a polypeptide from which recombinant gelatin can be derived, wherein such polynucleotide sequence is operably linked to a promoter to effect the expression of the polypeptide. In a further embodiment, the method additionally comprises co-expression of at least one polynucleotide sequence encoding an enzyme that catalyzes a post-translational modification, or subunit thereof, wherein such polynucleotide sequence is operably linked to a promoter. In these methods, the recombinant gelatins or collagenous polypeptides are extracted from the biomass.

20

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#### Fungi

- 30 Filamentous fungi may also be used to produce the polypeptides of the instant invention. Vectors for expressing and/or secreting recombinant proteins in filamentous fungi are well known in the art, and one of skill in the art could, using methods and products available in the art, use these vectors in the presently recited methods. (See, e.g., U.S. Patent No. 5,834,191.)

#### Insect

- 35 Insect cell systems allow for the polypeptides of the present invention to be produced in large quantities. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in, for example, *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. Sequences encoding the gelatins or gelatin precursors of the present



5 invention may be cloned into non-essential regions of the virus, for example, the polyhedron gene, and placed under control of an AcNPV promoter, for example, the polyhedron promoter. Successful insertion of a coding sequence will result in inactivation of the polyhedron gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat encoded by the polyhedron gene). These recombinant viruses are then  
10 used to infect *Spodoptera frugiperda* cells or *Trichoplusia* larvae in which polynucleotides encoding the gelatins or gelatin precursors are expressed. (See, e.g., Engelhard, E. K. et al. (1994) Proc. Nat. Acad. Sci. 91:3224-3227; Smith et al. (1983) J. Virol. 46:584; and U.S. Patent No. 4,215,051). Further examples of this expression system may be found in, e.g. Ausubel et al. (1995), *supra*.

15 Recombinant production of the polypeptides of the present invention can be achieved in insect cells, for example, by infection of baculovirus vectors containing the appropriate polynucleotide sequences, including those encoding any post-translational enzymes that might be necessary. Baculoviruses are very efficient expression vectors for the large-scale  
20 production of various recombinant proteins in insect cells. Various methods known in the art can be employed to construct expression vectors containing a sequence encoding a gelatin or gelatin precursor of the present invention and the appropriate transcriptional/translational control signals. (See, e.g., Luckow et al. (1989) Virology 170:31-39; and Gruenwald, S. and J. Heitz (1993) Baculovirus Expression Vector System: Procedures & Methods Manual,  
25 Pharmingen, San Diego, CA.)

#### Animal

The present invention provides methods of expressing the recombinant gelatins of the present invention, or polypeptides from which the recombinant gelatins of the present invention can  
30 be derived, in animal systems. Such systems include mammalian and non-vertebrate host cells and transgenic animals. In mammalian host cells, a number of expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding the polypeptides of the present invention may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader  
35 sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion into a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing the polypeptides of the present invention in infected host cells. (See, e.g., Logan, J. and Shenk, T. (1984) Proc. Natl. Acad. Sci. 81:3655-3659.) Alternatively, the vaccinia 7.5 K promoter may be used. (See, e.g.,

- 5     Mackett et al. (1982) Proc. Natl. Acad. Sci. USA 79:7415-7419 (1982); Mackett et al. (1984),  
J. Virol. 49:857-864; and Panicali et al., (1982) Proc. Natl. Acad. Sci. USA 79:4927-4931.)

In addition, various transcription enhancers known in the art, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in, for example, mammalian host cells.

- 10    Semliki Forest virus is a preferred expression system as the virus has a broad host range such  
that infection of mammalian cell lines will be possible. Infection of mammalian host cells,  
for example, baby hamster kidney (BHK) cells and Chinese hamster ovary (CHO) cells, using  
such a viral vector can yield very high recombinant expression levels. More specifically, it is  
contemplated that Semliki Forest virus can be used in a wide range of hosts, as the system is  
15    not based on chromosomal integration, and therefore will be a quick way of obtaining  
modifications of the recombinant gelatins in studies aimed at identifying structure-function  
relationships and testing the effects of various hybrid molecules. Methods for constructing  
Semliki Forest virus vectors for expression of exogenous proteins in mammalian host cells are  
known in the art and are described in, for example, Olkkonen et al. (1994) Methods Cell Biol  
20    43:43-53.

- Additionally, CHO cells deficient in dihydrofolate reductase (dhfr) can be transfected with an  
expression plasmid containing a dhfr gene and the desired polynucleotide. Selection of CHO  
cells resistant to increasing concentrations of methotrexate will undergo gene amplification,  
25    providing higher expression levels of the desired recombinant protein, as known in the art.

- Transgenic animal systems may also be used to express the recombinant gelatins of the  
present invention or the polypeptides from which these recombinant gelatins can be derived.  
Such systems can be constructed, for example, in mammals by operably linking an encoding  
30    polypeptide to a promoter and other required or optional regulatory sequences capable of  
effecting expression in mammary glands. Likewise, required or optional post-translational  
enzymes that effect post-translational modifications, may be produced simultaneously in the  
target cells employing suitable expression systems. Methods of using transgenic animals to  
recombinantly produce proteins are known in the art. (See, e.g., U.S. Patent No. 4,736,866;  
35    U.S. Patent No. 5,824,838; U.S. Patent No. 5,487,992; and U.S. Patent No. 5,614,396; and co-  
pending U.S. Application Serial No. 08/987,292.)

5    **Vaccine Formulations**

The present invention specifically contemplates the use of the recombinant gelatins of the present invention as components of various pharmaceutical products, including vaccines. (See, e.g., co-pending, commonly-owned application U.S. Application Serial No. \_\_\_\_\_, entitled "Recombinant Gelatins," filed 10 November 2000, incorporated by reference herein  
10 in its entirety.) Therefore, in one aspect, the present invention provides a vaccine formulation comprising recombinant gelatin. In a preferred embodiment, the recombinant gelatin is derived from human sources. The present recombinant gelatins offer an advantage previously unavailable in the art: that of using gelatins derived from native human collagen sequence, thus reducing the risk of immunogenicity and/or antigenicity from the gelatin itself. In a  
15 specific embodiment of the present invention, a vaccine comprising a non-immunogenic recombinant gelatin is provided. (See Example 12.) In a further embodiment, the non-immunogenic recombinant gelatin is derived from human sequence. In other embodiments, the non-immunogenic recombinant gelatin is derived from animal sources, e.g., from bovine or porcine sequences. In further embodiments, the non-immunogenic recombinant gelatin  
20 comprises a non-native sequence.

In addition, as the present gelatins are produced recombinantly in a controlled environment, the risk of infectivity, from infective agents such as TSEs, pathologic bacteria, or viruses, or the risk of exposure to endotoxins and pathogens introduced during or left over from  
25 processing, are virtually eliminated. It is another object of the invention to provide improved stabilizing agents which include recombinant gelatins, and derivatives, fragments, or functional equivalents thereof, each with physical and chemical characteristics optimized for particular applications.

30 Stability is a critical issue in vaccine development, where the relative instability of most existing viruses must be addressed. Vaccines can degrade over time, losing potency and effectiveness. In addition, vaccines are distributed worldwide, and in a diverse range of environments and conditions. Delivery of vaccines in developing countries, for example, has sometime been problematic due to imperfect storage conditions, including, e.g., high ambient  
35 temperatures. Under certain storage and/or transport conditions, the stability of a vaccine formulation can be compromised. (See, e.g., Chang, A. C. et al. (1996) J. Pharm. Sci. 85:129-132; Koyama, K. et al. (1996); Osame, J., European Patent No. 0 568 726 B1; and U.S. Patent No. 4,337,242.)

5 Vaccine stabilization refers to the maintenance of the safety and effectiveness of a vaccine. Stabilizing agents are thus compounds that perform a variety of different tasks. For example, with live vaccines, such as those for measles, mumps, rubella, and canine distemper, stability involves maintenance of the infectious titer/infectivity of the vaccine and the ability to elicit the appropriate immune response. With inactivated vaccines, such as those for influenza, 10 Hepatitis A, poliomyelitis (IPV), and rabies, as well as in vaccines produced from purified bacterial protein toxins, such as against cholera and pertussis, the ability to induce the desired immune response requires maintenance of the structural integrity of the vaccine, in order to preserve antigenic structure and correct steric presentation of relevant epitopes. Therefore, there is a need for effective stabilization of vaccine preparations in anticipation of varying 15 lengths of storage, and varying conditions of storage, transport, and use.

Current stabilization methods seek to improve storage and heat stability, boost immunological responses to the infectious agent by ensuring proper *in vivo* delivery, and provide formulations having the potential for reducing the number of immunizations required for 20 protective efficacy. Several stabilization methods, such as the use of low temperatures, lyophilization processes, and chemical stabilizers, have been used in the past. Low temperature storage facilities, for example, offering storage conditions of from about -10°C to -70°C, can provide one method of maintaining the stability of vaccine formulations. However, such facilities are not always available during the transport or distribution of 25 vaccines. Maintaining a proper "cold chain" of storage facilities can be difficult in view of the proper infrastructure required to monitor and maintain the necessary equipment, and to protect the vaccine formulation from development through to administration.

Another stabilization technique involves the use of various lyophilization or freeze-drying 30 procedures. Lyophilization is a reasonably effective but expensive procedure. The lyophilization process requires three distinct stages (freezing, primary drying, and secondary drying), each of which presents challenges for maintaining the native conformation and activity of biological macromolecules and microorganisms. (See, e.g., Burke et al. (1999) Crit. Rev. Ther. Drug Carrier Syst. 16:1-83, and references therein.) Lyophilized vaccines 35 remain fairly stable at about 4° to 8°C, but undergo deterioration throughout the storage period. Lyophilized vaccines slowly deteriorate until, at around 12 to 24 months of storage, the vaccine formulation lacks sufficient titer to confer immunization. In addition, lyophilized vaccine formulations must be reconstituted prior to use. Furthermore, the liquid reconstituted preparation loses potency while at room temperature. This can result in an imperfect immune

- 5 response, and can lead to an increase in the number of immunizations required for protective efficacy.

In another approach to confer stability, stabilizing agents are added to a vaccine formulation and used in conjunction with either lower temperature storage or lyophilization methods.

- 10 These stabilizers can perform a variety of functions directed to maintenance of stability, including, for example, maintaining formulation pH, contributing to viscosity and/or tonicity, assisting in controlled release, minimizing aggregation and precipitation of formulation components, and hydrating formulation components, to prevent damage caused by dehydration during desiccation and/or subsequent long-term storage, which can include
- 15 preferential hydration of protein constituents to maintain native protein conformation. In order to maximize the stabilizing effects of these materials, stabilizers are often employed in combination, so that the stabilizing effect can be synergistically or additively increased.

- Stabilizers used in the formulation of vaccines thus include, but are not limited to, buffers;
- 20 salts, such as sodium chloride or magnesium chloride; amino acids, such as sodium glutamate, arginine, lysine, and cysteine; monosaccharides, such as glucose, galactose, fructose, and mannose; disaccharides, such as sucrose, maltose, and lactose; sugar alcohols, such as sorbitol, and mannitol; polysaccharides, such as oligosaccharides, starch, cellulose, and derivatives thereof; human serum albumin and bovine serum albumin; various
- 25 antioxidants, such as ascorbic acid; and gelatin, such as hydrolyzed gelatin.

Hydrolyzed gelatin has been employed as a component of vaccines, for example, as a stabilizing agent for liquid and lyophilized vaccine formulations. (See, e.g., U.S. Patent No. 4,985,244; U.S. Patent No. 4,147,772; U.S. Patent No. 4,338,335; and Makino, S. et al.

- 30 European Patent No. 0 295 043 B1.) Partially hydrolyzed gelatins currently available are derived from gelatins produced by conventional extraction methods from raw animal sources. (See, e.g., Asghar, A. (1982) Adv. Food Res. 28:231-372; and Miller, E. J. et al. (1982) Methods in Enzymology 82:33-64.)

- 35 Gelatin can serve to stabilize vaccines, for example, lyophilized vaccines, at different stages. Lyophilization involves various extremes, including extremes of temperature, pH, salt concentration, etc. Redox reactions can occur as the solvent is removed, and the solutes are concentrated and dried. Various characteristics of gelatin, including its surface-active, gluing,

- 5 emulsifying, and viscosity-enhancing properties, can contribute to its stabilizing effects when used in such circumstances.

While lyophilized animal-source gelatins have been used with some success, these are essentially proteins foreign to the human system, and therefore capable of causing undesirable  
10 antigenic and/or immunogenic responses. Moreover, currently available materials are plagued by problems of variability and purity. Concern over the presence of infective agents such as TSEs in animal-derived proteins has led to governmental and regulatory restrictions relating to the recovery and use of such materials. For example, endotoxin levels of commercial materials typically range from about 1.0 to 1.5 EU/mg of gelatin. (See, e.g., Schaeffer, H.  
15 and G. von Jagow (1987) *Anal. Biochem.* 166:368-379; and Friberger, P. et al. (1987) in "Detection of Bacterial Endotoxins with the Limulus Amebocyte Lysate Test," *Prog. Clin. Biol. Res.* 231:149-169.)

The present invention provides recombinant gelatins that can be used as a component of  
20 vaccines, and methods for producing such recombinant gelatins. In one aspect, the recombinant gelatin is human recombinant gelatin. In the methods of the present invention, the endotoxin levels can be reduced by two to three orders of magnitude from those of commercially available gelatins derived from animal sources. (See Example 8.) The present invention thus provides, in one embodiment, a recombinant gelatin that is virtually endotoxin-  
25 free.

Furthermore, the present invention specifically provides a non-immunogenic gelatin. In one embodiment, the present invention provides for vaccine comprising recombinant gelatin, wherein the recombinant gelatin is human gelatin. Use of such a material as a component in  
30 vaccine formulations can reduce the risk of immunogenicity currently presented by the use of animal-derived gelatin components. (See, e.g., Sakaguchi and Inouye, *supra*; Sakaguchi *et al.*, *supra*; Nakayama *et al.*, *supra*; Asher, *supra*; and Verdrager, *supra*.) The use of gelatins derived from native human sequences will reduce the likelihood of a post-administration immunogenic response to the gelatin product. In one embodiment, the present invention  
35 provides for the use of recombinant gelatins encoded by altered collagen constructs, wherein the encoded gelatins are non-immunogenic. (See Example 12) Such recombinant gelatins can be constructed using assays designed to identify regions responsible for inducing immunogenic and antigenic responses, and the recombinant gelatins of the present invention can be specifically tailored to avoid these domains.

5 In addition to providing a gelatin material without the antigenicity, immunogenicity, and infectivity risks associated with the use of animal-derived materials, the present invention allows for a reproducible source of consistent product. Specifically, the present gelatins can be presented as a homogenous mixture of identical molecules. The physical characteristics desired in a particular medical application can be specifically introduced and achieved  
10 consistently. The present invention is thus able to provide a reliable and consistent product will minimize variability associated with the availability and use of current gelatin products.

The recombinant gelatins of the present invention can be designed to possess specific physical properties suitable for use in particular applications. The present invention provides methods  
15 for varying characteristics such as molecular weight, gel strength, and pH of the final gelatin formulation to produce gelatins with specific properties as desired, and to thus meet customer's specifications to a degree unattainable with currently available materials. Moreover, such formulations allow the customer to explore refinements of existing processes and formulations, as well as to develop new applications, for the present recombinant gelatins.

20 With respect to the problem of purity, the present invention provides recombinant gelatins that are virtually endotoxin-free. (Example 8.) Furthermore, risks associated with variability and associated problems in reproducibility and predictability of behavior, are virtually eliminated as the recombinant gelatins of the present invention can comprise gelatin  
25 polypeptides with well-defined characteristics and behaviors. In one embodiment, the present invention provides a composition comprising a homogenous mixture of recombinant gelatin polypeptides. In a further embodiment, the present invention provides a heterogeneous mixture of recombinant gelatin polypeptides.

30 In one embodiment, the invention encompasses recombinant gelatins with specific molecular weights. In another embodiment, the present invention provides for recombinant gelatins possessing particular ranges of molecular weights. (See Example 1.) For example, the present invention allows for the production of recombinant human soluble gelatins with molecular weight distributions similar to those of currently available animal-derived soluble  
35 gelatins used in the formulation of vaccines as stabilizers. (See Examples 1, 7, and 9.)

Recombinant gelatins comprising uniform polypeptides of specific molecular weights are specifically provided. Gelatins currently used in vaccines are typically hydrolyzed fragments of approximately 60 kDa and less. Such fragments are preferred, for example, to reduce the

5 risk of immunogenic response to the gelatin. The present gelatins can be provided as small molecular weight polypeptides. In addition, using the methods of the present invention, specific non-immunogenic recombinant gelatins can be constructed, by identifying regions in a collagen or collagenous sequence responsible for inducing an immunogenic response, and producing a recombinant gelatin that does not contain that region. (See, e.g., Example 12.)

10

It is to be noted that gelatin is used in the processing and preparation of vaccines, as well as appearing as a component of vaccine formulations. The recombinant gelatins of the present invention can be used in preparation and processing steps, and provide distinct advantages over currently used animal-derived products. For example, the gelatins of the present invention can be produced as consistent molecules with defined and characterized properties,

15

limiting variability in performance and allowing for fine-tuning and reproducibility of manufacturing procedures.

The molecular weight distributions of commercially available animal-derived soluble gelatins, such as those used in formulation of vaccines, range from about 0 to 30 kDa and from about 0 to 60 kDa. (See Example 9.) The present invention provides a method of producing recombinant human gelatins with molecular weight distributions that correspond to those of commercially available gelatins, and can be used for the same purposes. Additionally, the present invention provides methods for producing gelatins with a narrower molecular weight distribution, for example, about 10 to 30 kDa, or about 30 to 50 kDa, not available from commercial materials.

20

25

The present invention provides multiple methods of producing the recombinant gelatin of the appropriate molecular weight for use in a certain application. For example, recombinant gelatin can be derived through processing of recombinant collagen, such as by hydrolysis techniques, including acid, thermal, or enzymatic hydrolysis. (See Examples 9 and 10.) In another aspect, the recombinant gelatin is non-hydrolyzed, and the recombinant gelatin of the appropriate size is obtained through expression of recombinant gelatin from an altered collagen construct. (See Example 1.)

30

35

The present invention provides for the production of recombinant gelatins specifically tailored, through manipulation of cross-linking, hydroxylation, molecular weight, or any combination thereof, to possess defined thermal characteristics suited for particular applications. In one aspect, the invention provides recombinant gelatin, suitable for use as a



5 stabilizing agent or excipient for vaccines, that improves thermal stability, even when these vaccines are subjected to high temperatures for long periods. In another aspect, the present invention provides stabilizing agents suitable for use in association with certain vaccines whose active components are thermo-sensitive, including viral vaccines such as, for example, yellow fever. In one embodiment, the present invention provides a recombinant gelatin that  
10 stabilizes vaccines at above sub-zero temperatures.

It is to be noted that the present invention specifically contemplates that the present recombinant gelatins can provide an acceptable source of material for use by populations whose religious or other dictates preclude the use of materials derived from particular animal  
15 sources, e.g., from bovine or from porcine sources.

In one embodiment, the vaccines of the present invention can be manufactured in ready-to-use form, such as in filled syringes or in microencapsulated form, eliminating the need for subsequent processing prior to use.

20 Hydrolyzed gelatin is used as a stabilizer in current vaccine formulations. The present invention provides in one embodiment recombinant gelatin with characteristics similar to those of hydrolyzed animal-source gelatin, e.g., similar molecular weights, melting temperatures, etc. These recombinant gelatins can be produced through hydrolysis of  
25 recombinant collagen, or can be produced directly from altered collagen constructs. The present invention provides gelatins that are reproducible and well-characterized proteins with defined properties. Therefore, the recombinant gelatins used, in the preparation and manufacture of vaccines and/or as stabilizers and components of vaccines, can be produced according to the present methods to have controlled and particular characteristics. Use of the  
30 present gelatins thus permits vaccine makers an opportunity to fine-tune and minimize variability in their production processes, as well as to use recombinant gelatin in a more effective and more cost-efficient manner than currently available animal-source products are used.

35 It is specifically contemplated that the recombinant gelatins of the present invention can be used in any type of vaccine. For example, the present recombinant gelatins can be used in live attenuated, inactivated, and subunit vaccines, in single-dosage and combination vaccines, in virus-based, bacterial-based, parasite-based, and nucleic acid vaccines, including synthetic and semi-synthetic vaccines.

5

The present vaccines can be formulated in any fashion appropriate for the particular vaccine, for example, as liquid or as freeze-dried formulations. More particularly, the invention relates to compositions that stabilize liquid and lyophilized viral vaccines and protein-based pharmaceuticals. Different types of vaccines, directed to various microorganisms, including  
10 viruses, bacteria, and parasites, etc., are contemplated. For example, the recombinant gelatins of the present invention can be used in the formulation of, e.g., live, inactivated, and subunit vaccines.

In one aspect, the present invention provides a subunit vaccine comprising recombinant  
15 gelatin. In a further embodiment, the subunit vaccine comprises non-immunogenic recombinant gelatin. In one embodiment, the recombinant gelatin can be recombinant human gelatin. In various embodiments, the recombinant gelatin is derived from hydrolysis of recombinant collagen, is produced directly from altered collagen constructs, is a homogenous mixture of uniform molecular weight, and has the melting temperature most appropriate for  
20 the vaccine's particular formulation and delivery mechanism. In another embodiment, the recombinant human gelatin comprises a sequence selected from the group consisting of SEQ ID NOs:15 through 25, 30, 31, and 33.

The recombinant gelatins of the present invention can be used as stabilizers in any vaccine  
25 directed to any infective agent. Examples of such vaccines include, but are not limited to, vaccines for vaccinia virus (smallpox), polio virus (Salk and Sabin), mumps, measles, rubella, diphtheria, tetanus, Varicella-Zoster (chicken pox/shingles), pertussis (whooping cough), Bacille Calmette-Guerin (BCG, tuberculosis), haemophilus influenzae meningitis, rabies, cholera, plague, Japanese encephalitis virus, salmonella typhi, shigella, hepatitis A, hepatitis  
30 B, rotavirus, adenovirus, yellow fever, foot-and-mouth disease, herpes simplex virus, respiratory syncytial virus, rotavirus, Dengue, West Nile virus, Turkey herpes virus (Marek's Disease), influenza, parainfluenza, respiratory syncytial virus (RSV), typhus, pneumonia, Lyme disease, and anthrax. The term vaccine as used herein includes reference to vaccines to various infectious and autoimmune diseases and cancers that have been or that will be  
35 developed, for example, vaccines to various infectious and autoimmune diseases and cancers, e.g., vaccines to human immunodeficiency virus (HIV), hepatitis C virus (HCV), and malaria, and vaccines to breast, lung, colon, renal, bladder, and ovarian cancers.

- 5 The present invention encompasses formulations intended for various types of delivery. The vaccines can be formulated for injection, including, for example, subcutaneous, parenteral, e.g., intramuscular, and intravenous injection. In one embodiment, the vaccine is formulated for delivery to a mucosal surface, such as for oral or nasal delivery, in spray, liquid, or other forms. In a further aspect, such formulations include mucosal absorption enhancers as
- 10 appropriate. Vaccines formulated as inhalants, e.g., for deep lung delivery, are specifically contemplated. Such a vaccine could be formulated, for example, in fine powder form. The vaccines of the present invention could also be formulated for transdermal or liposomal delivery. Various formulations suitable for, e.g., enteric release are specifically contemplated.
- 15 The vaccine formulations of the present invention can include vaccines intended as edible vaccines, such as food vaccines, in which the antigen is delivered in edible form, such as in transgenic plants, e.g., potatoes, is capable of inducing the appropriate protective response. (See, e.g., Tacket, C. O. *et al.* (2000) *J. Infect. Dis.* 182:302-305; and Richter, L. J. *et al.* (2000) *Nat. Biotech.* 18:1167-1171.) The vaccines of the present invention can be provided in
- 20 the form of transgenic plants, e.g., edible fruits, such as bananas or strawberries.

Encapsulation of the present invention, for example, in microspheres, is specifically contemplated. (See, e.g., Moldoveanu, Z. *et al.* (1993) *J. Inf. Dis.* 167:85-90.) Controlled release technologies available to those in the art can be applied to obtain formulations

25 appropriate in, for example, various release forms, such as, e.g., transdermal, pulmonary, and polymeric release forms are specifically contemplated.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration and parenteral delivery, including intramuscular, subcutaneous,

30 intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. Formulations for enteric release, etc., are also contemplated. The composition may be administered in a local rather than a systemic manner.

- 35 Techniques for various formulations and drug delivery systems are available in the art and are described in numerous sources. (See, e.g., Remington's Pharmaceutical Sciences, *supra*.) The most effective and convenient route of administration and the most appropriate formulation for a particular situation can be readily determined by methods known in the art.

- 5 The following examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

### 15 EXAMPLES

Unless otherwise stated, the following materials and methods were used in the examples of the present invention.

#### Example 1: Direct Expression of Recombinant Gelatins

- 20 Specific fragments of the  $\alpha 1(I)$  cDNA from human type I collagen were amplified by PCR and cloned into the plasmid pPICZ $\alpha A$  or pPIC9K (Invitrogen Corp., Carlsbad, CA). The specific PCR primers used in cloning are set forth in Table 1 below. Specific recombinant gelatins are identified in Table 2 as SEQ ID NOs: 15 through 25, and 30, 31, and 33. These recombinant gelatins are additionally identified by reference to human prepro- $\alpha 1(I)$  collagen. (Genbank Accession No. CAA98968.) The expression plasmids used contained  $\alpha 1(I)$  cDNA sequences of different sizes fused to the yeast mating factor alpha prepro secretion sequence. Other signal sequences known in the art can also be used, for example, the yeast invertase (SUC2), the yeast acid phosphatase (PHO) sequences, the native pro-collagen signal sequence, and the signal sequence for human serum albumin. A signal sequence that provides the optimal level of expression for a specific gelatin fragment in a specific expression system should be chosen.

TABLE 1

SEQ ID NO:	SEQUENCE
1	GTATCTCTCGAGAAGAGAGAGGCTGAAGCTGGTCTGCCTGGTGCCAAGGGT
2	TAGACTATTATCTCTCGCCAGCGGGACCAAGCAGG
3	GTATCTCTCGAGAAGAGAGAGGCTGAGGCTGGAGCTCAGGGACCCCTGGC
4	ATGCTCTAGATTACTTGTCAACAGGGGCACCAAGCAGG
5	GTATCTCTCGAGAAGAGAGAGGCTGAAGCTGGCCCCATGGGTCCCTCTGGT CCT
6	TGCTCTAGATCATTAAGCATCTCCCTGGCACCATCCAA
7	TGCTCTAGACTATTAAAGGCGCGCCAGCATCACCCCTAGCACCATC

8	TGCTCTAGATCATTAAAGGCGCGCCAGGTTACCGCTGTTACCCCTTGGG
9	TGCTCTAGATCATTATCTCTCGCCTCTTGCTCCAGAGGG
10	GTGCCCGTGGTCAGGCTGGTGTGATGGGATTCCTGGACCTAAAGGTGCTGCTTAAT
11	CTAGATTAAGCAGCACCTTTAGGTCCAGGGAATCCCATCACACCAGCCTGACACGGGCAACCAG
12	ATGCTCTAGATTATTAAGGAGAAACCGTCTCGTCCAGGGGA
13	CTAGTCTAGATTATCTTCTCCAGAGGGGCCAGGGGC
14	CTAGTCTAGATTAGCGAGCACCTTGGCTCCAGGAGC
32	AGCTTCTAGATTATTAGGGAGGACCAGGGGGACCAGGAGGTCC

5

TABLE 2.

SEQ ID NO:	PCR PRIMERS USED	AMINO ACID SEQUENCE	MOLECULAR WEIGHT
15	SEQ ID NO:5 and SEQ ID NO:6	residue 179 to residue 280	9,447 Da
16	SEQ ID NO:5 and SEQ ID NO:8	residue 179 to residue 439	23,276 Da
17	SEQ ID NO:5 and SEQ ID NO:9	residue 179 to residue 679	44,737 Da
18	SEQ ID NO:10 and SEQ ID NO:11	residue 531 to residue 589	5,250 Da
19	SEQ ID NO:1 and SEQ ID NO:2	residue 531 to residue 631	8,947 Da
20	SEQ ID NO:1 and SEQ ID NO:7	residue 531 to residue 715	16,483 Da
21	SEQ ID NO:1 and SEQ ID NO:4	residue 531 to residue 781	22,373 Da
22	SEQ ID NO:1 and SEQ ID NO:12	residue 531 to residue 1030	44,216 Da
23	SEQ ID NO:3 and SEQ ID NO:7	residue 615 to residue 715	8,213 Da
24	SEQ ID NO:3 and SEQ ID NO:4	residue 615 to residue 781	14,943 Da
25	SEQ ID NO:3 and SEQ ID NO:12	residue 615 to residue 1030	36,785 Da
30	SEQ ID NO:3 and SEQ ID NO:13	residue 615 to residue 676	5,517 Da
31	SEQ ID NO:3 and SEQ ID NO:14	residue 615 to residue 865	22,126 Da
33	SEQ ID NO:1 and SEQ ID NO:32	residue 531 to residue 1192	~65 kDa

10 The expression plasmids were introduced into *Pichia pastoris* cells by electroporation, and transformants were selected by complementation of a *his4* auxotrophy (pPIC9K vectors) or by resistance to zeocin (pPICZαA vectors). Recombinant protein expression was regulated by the methanol-inducible alcohol oxidase promoter ( $P_{AOX1}$ ). The *Pichia pastoris* host cells contained integrated copies of the  $\alpha$  and  $\beta$  subunits of human prolyl 4-hydroxylase (P4H), the enzyme responsible for the post-translational synthesis of hydroxyproline in collagen, and

15 have been previously described. (See, e.g., Vuorela, M. et al. (1997) EMBO J 16:6702-6712.)

The yeast strains were grown in buffered minimal glycerol media, and recombinant protein expression was induced using the same media with methanol (0.5%) substituted for glycerol as the carbon source, as described in the Invitrogen *Pichia* Expression Manual. Gelatin-

20 producing strains were identified by 10-20% Tricine SDS-PAGE analysis of conditioned media and prolyl 4-hydroxylase activity in extracts from shake flask cultures. Co-expression of prolyl 4-hydroxylase and the collagen fragments resulted in the expression of recombinant gelatins with native human sequences.

5 The fragments were expressed and secreted into the media. Recombinant gelatin was recovered and purified from the media by acetone precipitation, anion or cation exchange chromatography, or any combination thereof. Acetone precipitation was performed at 4°C by addition of acetone to cell-free culture supernatants to a final concentration of 40%. The resulting precipitate, consisting primarily of endogenous yeast proteins, was collected by  
10 centrifugation. Acetone was then added to this supernatant to a final concentration of 80%, causing the gelatin to precipitate, which was then collected by centrifugation, dialyzed overnight against water, and lyophilized. High purity gelatin was obtained by a combination of anion and cation exchange chromatography. Chromatographic purifications were performed at room temperature.

15 Estimation of the sizes of collagenous proteins by electrophoresis, compared to calculation of molecular weight based on amino acid composition, is known in the art (Butkowski et al. (1982) Methods Enzymol 82:410-423) N-terminal sequence analysis of the recombinant gelatins demonstrated correct processing of the prepro sequence which was fused to the  
20 gelatin fragments in order to direct the protein to the yeast secretory pathway. The gelatins produced in this system contained only sequences derived from human collagen. Additionally, the recombinant gelatins represented the major component of the conditioned media, as *Pichia pastoris* cells secrete very few proteins.

25 The expressed recombinant gelatins were of discrete sizes, ranging from about 5 kDa to about 65 kDa as measured on SDS-PAGE, corresponding to gelatins of ~5 kDa (lane 2, SEQ ID NO:18), ~10 kDa (lane 3, SEQ ID NO:19), ~16 kDa (lane 4, SEQ ID NO:24), ~18 kDa (lane 5, SEQ ID NO:20), ~20 kDa (lane 6, SEQ ID NO:28) (also having a calculated molecular weight of 17,914 Da, not set forth in Table 1), ~33 kDa (lane 7, SEQ ID NO:27) (also having  
30 a calculated molecular weight of 29,625 Da, not set forth in Table 1), ~41 kDa (lane 8, SEQ ID NO:25), and ~50 kDa (lane 9, SEQ ID NO:22), as indicated in Figure 1 (lane 10 represents hydrolyzed recombinant human collagen type I, prepared as described in Example 10).

#### Example 2: Human Recombinant Gelatins Support Cell Attachment Activity

35 The recombinant human gelatin fragments of the present invention demonstrated *in vitro* cell attachment activity. In the following assay, 96-well Maxisorp plates (Nunc) were coated with the following recombinant human gelatin domains from the  $\alpha 1$  chain of human type I collagen, as described in Example 1 and listed in Table 2: SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22. VITROGEN bovine collagen (Cohesion Technologies;

5 Palo Alto, CA) and bovine serum albumin served as positive and negative controls, respectively. Each of the proteins was diluted to 0.1 mg/ml in 0.1 M NaHCO<sub>3</sub>, pH 10.0, and the plates coated overnight at 4°C. Human foreskin fibroblasts (HFF) or human umbilical vein endothelial cells (HUVEC, from Clonetics, passage 5), were seeded onto the coated plates and incubated for 60 minutes at 37°C. Experiments were performed in triplicate.

10

The degree of cell attachment was then measured using Reagent WST-1, the absorbance of which was read at 450 nm in an ELISA reader. Figure 2A shows that recombinant human gelatins supported HFF attachment to Maxisorp plates, and, for these cells, attachment was directly proportional to the molecular weight of the recombinant human gelatin coated in each well. Specifically, the recombinant gelatins of SEQ ID NO:19, SEQ ID NO:20, and SEQ ID NO:21 supported HFF attachment to a higher extent than that seen with BSA. Figure 2B shows that the different recombinant human gelatins supported endothelial cell attachment. Cell attachment activity was also demonstrated with recombinant human gelatin prepared by thermal hydrolysis of recombinant human collagen (described below in Example 9), using recombinant gelatins having molecular weight ranges of 0-30 kDa and 0-50 kDa.

20

#### Example 3: Identification of a Proteolytically Stable Gelatin Fragment

Recombinant gelatin fragments were found to be proteolytically modified during their expression and accumulation in the media of recombinant *Pichia pastoris* cells. Expression of several different portions of the helical domain of the  $\alpha 1$  chain of type I collagen lead to the identification of a recombinant gelatin that had superior stability with respect to proteolysis. Three different gelatin fragments were cloned into plasmid pPICZ $\alpha$ A, and their relative stabilities evaluated during recombinant protein expression in *Pichia pastoris* cells.

25

30 The first strain used is described above in Example 2, corresponding to SEQ ID NO:19. Additional strains were created using plasmids encoding human  $\alpha 1(I)$  helical domain amino acid residues 179-280 (SEQ ID NO:15) and 615-715 (SEQ ID NO:23). These recombinant gelatins were constructed as described in Example 1, using primers SEQ ID NO:5 and SEQ ID NO:6, and SEQ ID NO:3 and SEQ ID NO:7. The PCR products were digested with XhoI and XbaI, cloned, and prepared for electroporation as described above. The strains were grown, protein expression induced, and the expressed gelatin fragments compared by SDS-PAGE. Figure 3 shows that the recombinant gelatin of SEQ ID NO:15 (lane 2) and the recombinant gelatin of SEQ ID NO:19 (lane 3) underwent proteolysis, while the recombinant

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- 5 gelatin of SEQ ID NO:23 (lane 4) remained completely intact. This result demonstrated that recombinant gelatin fragments of the present invention could be produced which have superior stability.

Example 4: Expression of Hydroxylated Recombinant Human Gelatin

- 10 Prolyl 4-hydroxylase activity has not been detected in yeast. A *Pichia pastoris* strain has been engineered to express active prolyl 4-hydroxylase and has been used previously to produce hydroxylated collagen. (See U.S. Patent No. 5,593,859.) To express hydroxylated recombinant human gelatin, this strain was transformed with a gelatin expression cassette encoding 100 amino acids of a recombinant of human  $\alpha 1(I)$  collagen (SEQ ID NO:19, Table  
15 2), generated by PCR using the primers SEQ ID NO:1 and SEQ ID NO:2. The PCR DNA product (~330 bp) was digested with XhoI-XbaI and ligated into the XhoI-XbaI sites of pPICZ $\alpha$ A (Invitrogen), creating plasmid pDO7.

- A 1048 bp Cel II-AgeI fragment was isolated from pDO7 which contained the 3' portion of  
20 the AOX1 promoter region, the mating factor alpha secretion signal, the recombinant gelatin of SEQ ID NO:19, the polylinker sequence from pPICZ $\alpha$ A, and 56 base pairs of the AOX1 transcription terminator. This fragment was ligated into the Cel II-AgeI sites of pPIC9K (Invitrogen) to create pDO41. *Pichia pastoris* strain  $\alpha\beta 8$  (*his4*) was transformed with StuI-linearized plasmid pDO41 by electroporation, plated on minimal dextrose plates, and  
25 transformants were selected that complemented the *his4* auxotrophy. Approximately 20 *his*<sup>+</sup> transformants were grown and induced with methanol as described in Example 1. Strains that expressed SEQ ID NO:19 were identified by SDS-PAGE analysis of the conditioned media. (Figure 4.)

- 30 Recombinant gelatin fragments from positive strains were purified from the media by acetone precipitation, and analyzed further by amino acid analysis, as described, e.g., in Hare, PE. (1977) Methods in Enzymology 47:3-18. Amino acid analysis of the gelatin product from one of the strains demonstrated the presence of hydroxyproline in the secreted recombinant gelatins. The ratio of hydroxyproline to proline was determined to be 0.29 in gelatin isolated  
35 from the strain shown in shown in Figure 4, isolate #2, indicating co-expression of gelatin and prolyl 4-hydroxylase.



5 Non-hydroxylated recombinant gelatins were expressed and purified from a *Pichia pastoris* strain that does not express prolyl 4-hydroxylase. Proline residues within this recombinant gelatin were subsequently converted to hydroxyproline residues *in vitro* using prolyl 4-hydroxylase enzyme activity. A gelatin expression plasmid was created by PCR using primers SEQ ID NO:3 and SEQ ID NO:4, leading to the expression of recombinant gelatin of  
10 SEQ ID NO:24. The 525 base pair PCR product was purified and digested with XhoI-XbaI and ligated to XhoI-XbaI digested pPICZαA. The plasmid was linearized with PmeI and electroporated into *Pichia pastoris* strain X-33 (Invitrogen). Transformants were selected by growth on YPD plates containing 500 µg/ml zeocin. Strains were tested for gelatin expression as described above and recombinant non-hydroxylated gelatin was purified from  
15 the media of a positive isolate. Conditioned media was concentrated 10-fold by pressure dialysis using a 10 kDa molecular weight cut-off membrane, and the sample was dialyzed against Buffer A (50 mM Tris-HCl pH 9.0, 50 mM NaCl). The dialyzed material was chromatographed on a Q-sepharose column equilibrated in Buffer A. Gelatin does not bind to this column under these conditions, and therefore, was present in the flow-through fraction.  
20 The majority of the contaminating yeast proteins bound to the column and eluted with Buffer B (Buffer A + 0.5 M NaCl).

The flow-through fraction was dialyzed against 50 mM sodium acetate, pH 4.5, and the recombinant gelatin further purified on a SP-sepharose column equilibrated in the same  
25 buffer. The recombinant gelatin bound to the column, and was step-eluted with 0.2 M NaCl. The purified gelatin, at 1 mg/ml, was heat denatured (100°C for 10 minutes) and mixed with purified P4H at an enzyme to substrate ratio of 1:30 in the presence of the following components: 50 mM Tris-HCl pH 7.8, 2 mM ascorbate, 2 mM α-ketoglutarate, 0.1 mM FeSO<sub>4</sub>, 10 µM DTT, 10 mg/ml bovine serum albumin, and 100 units of catalase (Sigma  
30 Chemical Co., St Louis, MO). (See, e.g., Kivirikko, K.I. and Myllyla, R. (1982) *Methods in Enzymology* 82:245-304; and Vuori, K., et. al. (1992) *Proc. Natl. Acad. Sci.* 89:7467-7470.) The reaction was allowed to proceed at 37°C for 16 hours.

The recombinant gelatin was then purified by chromatography on Q-sepharose as described  
35 above. The bound proteins were eluted from the column with 0.5 M NaCl and collected. (Figure 5, lanes 7, 8, and 9.) The flow-through and eluate fractions were analyzed by SDS-PAGE to demonstrate the purity of the recovered gelatin. (Figure 5.) Amino acid analysis of the gelatin was performed following dialysis of the flow-through fractions. (Figure 5; lanes 3

- 5 through 6.) The amino acid analysis showed that the gelatin was 87% hydroxylated. Hydroxylation of 100% is achieved when the number of moles of hydroxyproline/moles of proline + moles of hydroxyproline in gelatin equals 0.5.

Example 5: Stability of Gelatins in the Presence or Absence of Prolyl 4-hydroxylase

- 10 An 18 kDa recombinant gelatin (SEQ ID NO:20) was expressed according to the methods described above. The expressed fragments were analyzed by gel electrophoresis. Recombinant gelatin expressed in the presence of prolyl 4-hydroxylase had markedly greater stability than the gelatin expressed in the absence of prolyl 4-hydroxylase. (See Figure 6.)

- 15 A role of proline hydroxylation on recombinant human gelatin stability and an enhancement of stability was found in prolyl 4-hydroxylase-expressing *Pichia pastoris* strains. A plasmid encoding SEQ ID NO:20 (pDO32) was constructed by PCR using primers SEQ ID NO:1 and SEQ ID NO:7. The PCR product was purified, digested, and cloned as described above. The same  $\alpha 1(I)$  cDNA fragment was expressed in host cells lacking prolyl hydroxylase, and in  
20 host cells containing the  $\alpha$  and  $\beta$  prolyl 4-hydroxylase subunits. Three *Pichia pastoris* strains were electroporated with PmeI-linearized pDO32: strain X-33 (wild-type *Pichia pastoris*), two prolyl 4-hydroxylase-expression strains: strain P4H-2, and strain  $\alpha\beta 8$ , as described in the U.S. Patent No. 5,593,859 and in Vourela et al. (1997) EMBO J 16:6702-6712.

- 25 Transformants were selected by resistance to 500  $\mu\text{g/ml}$  zeocin. Eight isolates from each transformation were grown and induced as described, and the stability of the expressed recombinant human gelatin was analyzed by SDS-PAGE. (See Figure 6.) In wild-type *Pichia pastoris* strain X-33, approximately equimolar amounts of intact recombinant gelatin and a proteolytic fragment (which migrated just below the recombinant gelatin on the gel,  
30 indicated by the arrow at the right of the figure) were observed. (Figure 6, strain X-33.) In both strains that co-express prolyl 4-hydroxylase, the amount of the proteolytic fragment was significantly reduced, demonstrating that co-expression of prolyl 4-hydroxylase along with recombinant human gelatin enhanced gelatin stability by substantially reducing proteolysis of the gelatin. (Figure 6, strain P4H-2 and strain  $\alpha\beta 8$ .)

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5    Example 6: Enhanced Recombinant Human Gelatin Expression by Supplementation of Expression Media

Previous reports have indicated that casamino acid-supplemented media decreased the amount of proteolysis seen during expression of certain proteins in *Pichia pastoris*. (Clare, J.J. et al. (1991) Gene 105:202-215.) The breakdown of the present recombinant human gelatin expressed in *Pichia pastoris* was measured following enrichment of the expression media with various supplements. In this particular study, the *Pichia pastoris* strain  $\alpha\beta 8$  described in Example 5, which expressed recombinant human gelatin fragment SEQ ID NO:20 was employed. (Example 5 and Table 2.) Recombinant gelatin was induced in media supplemented with a range of concentrations (0-2%) of various supplemental components, including casamino acids, casitone, yeast extract, peptone, peptamin, tryptone, Gelatone, lactalbumin, and soytone. Several formulations, including lactalbumin hydrolysate, soytone, casitone, and peptamin (Difco Laboratories, Detroit, MI) increased recombinant gelatin expression levels. (Figure 7, lactalbumin and soytone.)

20    These results indicate that specific media supplements employed during the expression of recombinant gelatins can lead to increased production. In one aspect, the use of soytone as a media supplement provided a plant-derived (rather than animal-derived) media component that lead to increased expression of recombinant gelatin. This would provide an animal material-free environment for production of recombinant gelatin that could be used in a variety of applications.

25    Example 7: Cross-linking of Recombinant Human Gelatins

A slurry of recombinant human collagen (obtained as described in U.S. Patent No. 5,593,859) was prepared by dissolving 10.8 mg of recombinant human collagen type I in 5 ml of water, followed by dialysis against 20 mM sodium phosphate, pH 7.2. The final recombinant human collagen concentration of the slurry was approximately 2 mg/ml. Preparation of cross-linked recombinant human gelatin was performed by adding 10  $\mu$ l or 5  $\mu$ l of a 20% solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce Chemical Co.) to 1 ml of the recombinant human collagen slurry described above. The cross-linking reaction occurred overnight at room temperature. Unreacted EDC was removed by dialysis against water.

The resulting cross-linked recombinant human gelatins were analyzed by 6% glycine SDS-PAGE analysis. Figure 8 shows an SDS-PAGE comparison of recombinant human gelatin

5 (lane 6, labeled UNL-5-4), cross-linked recombinant human gelatin (lane 5, labeled UNL 5-4, 0.1% EDC; lane 4, labeled UNL 5-4, 0.2% EDC), commercially available hard capsule gelatin (lane 3), and commercially available gelatin (Type A, from porcine skin, approximately 300 Bloom, lane 2) obtained from Sigma Chemical Co. As shown in the SDS-PAGE analysis of Figure 8, the commercial capsule gelatin and Sigma gelatin contained  $\alpha$ -  
10 chain (molecular weight of approximately 110 kDa) as a major component, as well as a smear of higher molecular weight gelatin components (with molecular weight ranging from approximately 200-250 kDa). The recombinant human collagen was composed of  $\alpha$ -chain only. Following cross-linking, however, the cross-linked recombinant gelatin was composed of  $\alpha$ -chain as well as a smear of higher molecular weight gelatins, similar to that observed in  
15 commercial gelatin and commercial capsule gelatin. This indicated that recombinant human gelatins displaying a molecular weight distribution similar to that of commercial capsule gelatins could be produced by cross-linking recombinant human collagen. Cross-linked recombinant gelatins would have use in applications in which increased gel strength and increased viscosity would be desirable.

20

Example 8: Endotoxin Levels of Commercially Available Gelatin and Soluble Recombinant Human Gelatin

Endotoxin levels of soluble gelatin obtained commercially from Kind & Knox (K&K) and the recombinant human gelatins of the present invention (made as described in Example 9) were  
25 determined using the Limulus Ameobocyte Lysate test, as known in the art. (See, e.g., Friberger, P. et al. (1987) Prog. Clin. Biol. Res. 231:149-169.) Three different gelatin concentrations were examined. As shown in Table 3, the recombinant human gelatins generated by thermal hydrolysis of recombinant human collagen type I (rhCI) of the present invention were virtually endotoxin-free. The endotoxin levels of commercially available  
30 materials were about 1 to 1.5 EU/mg of protein. The methods for producing gelatin as described in the present invention resulted in gelatins having substantially lower endotoxin levels, by two to three orders of magnitude, than those of the commercial preparations. Such low endotoxin levels make the recombinant gelatins of the present invention especially attractive for use in certain applications, such as use in pharmaceutical stabilization.

35

TABLE 3

Gelatin Concentration (mg/ml)	K&K Gelatin (EU/mg)	Recombinant Human Gelatin (EU/mg)
3	1.03	< 0.005
1.5	1.41	< 0.005
0.75	1.29	<0.006

Example 9: Derivation of Gelatins by Thermal and Acid Hydrolysis

Hydrolysis procedures (acid, thermal, and enzymatic) were developed to produce soluble recombinant human gelatins with molecular weight distributions similar to those of currently available soluble animal-derived gelatins, used, for example, as stabilizers in the formulation of vaccines. For these experiments, intact recombinant human collagen type I and type III were used as starting materials. By varying the hydrolysis conditions, it was possible to vary the molecular weights of the final materials, producing materials of defined molecular weights.

*Molecular weight distribution of commercially available gelatins:*

These recombinant human gelatins were compared against commercially available gelatins. Four low molecular weight gelatin samples produced by Leiner Davis, Great Lake, Kind & Knox, and Dynagel, were obtained for characterization. All gelatins examined were soluble at room temperature. The molecular weight distributions of the gelatins on a Tricine SDS-PAGE gel are shown on Figure 9 and listed in Table 4. The gel profiles indicated the molecular weight distributions of commercially available gelatins were approximately 0-55 kDa, with the exception of the Dynagel-1 sample, which had a molecular weight distribution of 0-30 kDa. The gel profiles also revealed two patterns of molecular weight distribution. In one example, derived from the samples from Leiner Davis and Great Lakes, several discrete molecular bands were observed by SDS-PAGE. The pattern in the second example, derived from the Dynagel and Kind & Knox samples, showed a continuous distribution of material on the gel, with no discrete banding. The molecular weight distributions of Dynagel-1 and Dynagel-2 were quite different, despite being produced by the same manufacturer for the same application. This result indicated that batch-to-batch variation could be quite significant in currently available gelatins.

5

TABLE 4

Company	Relative Mobility	Maximum Apparent Molecular Weight (Da)	Molecular Weight* Distribution (Da)
K & K	0.3410	70,000	0-55,500
Leiner Davis	0.3410	70,000	0-55,500
Great Lake	0.3693	60,000	0-47,600
Sol-U-Por, # 1	0.3483	65,000	0-51,600
Sol-U-Por, # 2	0.4972	37,000	0-29,400

\* The molecular weight was adjusted by a factor of 1.26, which is the ratio of the mean residue weight of the standard proteins (115) over the mean residue weight of the collagenous proteins (91.6).

10

Heat hydrolysis of gelatins was performed as follows. The commercially available dry gelatins were dissolved in 40°-50°C water to make a 5% gelatin solution. The pH of the solution was adjusted with either 0.1N NaOH or 0.1N HCl in preparation for heat hydrolysis. Both type I and type III recombinant human collagens were expressed in *Pichia pastoris* and purified, as described in U. S. Patent No. 5,593,859. The final recombinant human collagen was dissolved in 10 mM HCl, dialyzed against water, and lyophilized. The lyophilized recombinant human collagen was dissolved in 40°-50°C water to make a 3% solution. The pH of the solution was adjusted as indicated below prior to heat hydrolysis.

15

Heat hydrolysis was performed in 1 ml Reacti-Vials (Pierce). The hydrolysis temperature varied from 100°C to 150°C, depending on the experiment. The pH of the hydrolysis solution varied from pH 2 to pH 7, as indicated. The hydrolysis time was also varied from one to thirty-two hours, depending on the temperature and pH of the solution. The gelatin hydrolysates were sampled at various time intervals and analyzed by SDS-PAGE.

25

#### *Hydrolysis of Commercially Available Gelatins at 120 °C:*

Samples of high molecular weight gelatin from Sigma (Type A from porcine skin, 250 kDa) were dissolved in six different pH solutions (5% gelatin) and hydrolyzed at 120°C. The pH 2 and pH 3 solutions were hydrolyzed for two and a half hours and sampled every half hour.

30

The pH 4 solutions were hydrolyzed for five hours and sampled every hour. The pH 5, pH 6, and pH 7 solutions were hydrolyzed for 24 hours and sampled every two hours after 14 hours of hydrolysis.

The hydrolysis patterns were analyzed on Tricine 10-20% SDS-gels as shown in Figures 10A, 10B, 10C, 10D, 10E, and 10F. The gel profiles show that the lower the pH of the solution, the more quickly the hydrolysis of the gelatin occurred. The gel profiles also revealed two

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5 hydrolysis patterns among the hydrolysates. One pattern showed several discrete molecular bands on the gel (see the acid hydrolysis results of the pH 2 and pH 3 solutions, Figure 10A and 10B), while the other pattern showed a continuous distribution of material on the gel (see the hydrolysis results of the pH 4, pH 5, pH 6, and pH 7 solutions, Figure 10C, 10D, 10E, and 10F).

10

These results showed that the process outlined above, or variations thereof, produced two different types of material, as seen in the analysis of the commercially available gelatins (discrete bands vs. a continuous distribution of material on SDS-PAGE). These experimental results also indicated that heat degradation of high molecular weight gelatin generated various sizes of soluble gelatins. Table 5 shows the molecular weight distributions obtained using Sigma Gelatin, following hydrolysis at 120°C in pH 6.0 solution.

15

TABLE 5

20

Hydrolysis Time (hr)	Relative Mobility	Max. App. Mol. Weight (Da)	Molecular Weight Distribution (Da)
4	0.2356	140,000	0-111,000
8	0.2890	90,000	0-71,400
11.5	0.3372	75,000	0-59,500
16	0.3837	47,000	0-37,300
20	0.4186	40,000	0-31,700
24	0.4525	33,000	0-26,200

*Hydrolysis of Commercially Available Gelatins at 150 °C:*

Samples of high molecular weight gelatin from Sigma (Type A from porcine skin, 250 kDa) were dissolved in four different pH solutions (5% gelatin) and hydrolyzed at 150°C for up to ten hours. The hydrolysates were sampled every two hours for analysis. The hydrolysis patterns were analyzed by Tricine 10-20% SDS-PAGE gels as shown in Figures 11A, 11B, 11C, and 11D. The gel profiles indicated that the degradation of gelatin occurred much more rapidly at 150°C than at 120°C. Additionally, hydrolysis of gelatins performed at 150°C produced gelatin fragments of lower molecular weights. Table 6 shows the molecular weight distributions of Sigma Gelatin, following hydrolysis at 150°C in pH 6.0 solution.

30

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TABLE 6

Hydrolysis Time (hr)	Relative Mobility	Max. App. Mol. Weight (Da)	Molecular Weight Distribution (Da)
2.5	0.2833	95,000	0-75,400
4.5	0.4555	41,000	0-32,500
6	0.5277	32,000	0-25,400
8	0.5833	24,000	0-19,000
10	0.6611	15,000	0-11,900

Example 10: Acid and Thermal Hydrolysis of Recombinant Human Collagen I and III

Recombinant human collagen type I was hydrolyzed at 120°C for up to 8 hours under neutral pH conditions (pH 7), or up to 3 hours in acidic pH conditions (pH 2). Recombinant human collagen type III was also hydrolyzed at 120°C for up to six hours in both neutral and acidic conditions. Hydrolysis was performed as described in Example 9. The human recombinant type I and type III hydrolysates were analyzed by Tricine 10-20% SDS-PAGE gels, shown in Figures 12A and 12B. The SDS-PAGE gel patterns indicated that the heat hydrolysis of recombinant human collagen was identical to the hydrolysis patterns of high molecular weight gelatins derived from natural sources. (Figure 9, Figures 10A through 10F, and Figures 11A through 11D, to Figures 12A and 12B.) Similar to the hydrolysis of natural gelatins (pH 7), the acid hydrolysates of recombinant human collagen showed several discrete molecular weight bands, while the neutral hydrolysates showed a more continuous molecular weight distribution. The molecular weight distribution of the neutral hydrolysates of recombinant human gelatin was around 0-70 kDa after six to eight hours of heat degradation. The hydrolysis under acidic conditions occurred much faster. The molecular weight distributions of the acidic hydrolysates of recombinant human gelatin were much narrower, around 0-10 kDa, after two to three hours of heat treatment.

25

As a further refinement of the heat hydrolyzed recombinant human gelatins discussed, we have demonstrated the utility of a yeast multi-gene recombinant expression methodology for the production of human gelatins with discrete fragments of the  $\alpha 1$  (I) chain of human type I collagen. This technology allowed us to produce well-defined, highly homogenous gelatin fragments ranging in size from 6-65 kDa. This presents unsurpassed flexibility in terms of the size and biophysical properties of the gelatin that can be used for specific applications.

30



5 Example 11: Enzymatic Hydrolysis of Recombinant Human Collagen Type I

Recombinant human collagen type I was hydrolyzed enzymatically, using the proteases set forth in Table 7. Recombinant human collagen type I was incubated with each enzyme at 37°C, using a substrate to enzyme ratio (w/w) as indicated in Table 7. The human recombinant type I hydrolysates obtained by treatment were analyzed by Tricine 10-20% SDS-PAGE gels. The results obtained from papain and protease X treatment are shown in Figure 13. The SDS-PAGE gel patterns indicated that the enzymatic hydrolysis of recombinant human collagen lead to different molecular weight distributions of the gelatins. Enzymatic hydrolysis using papain resulted in a continuous hydrolysis pattern, as indicated in Figure 13 and in Table 7, while hydrolysis using protease X resulted in several discrete molecular weight bands. As indicated in Table 7, the recombinant gelatins produced by this method had different hydrolysis patterns as a result of the particular enzymatic hydrolysis treatment. This presents great flexibility in producing sizes and biophysical properties of the gelatin that can be used for specific applications.

20

TABLE 7

Enzyme	Enzyme Activity / mg Protein	Substrate to Enzyme Ratio	Hydrolysis Pattern
Chymo-papain	1 U @ 37°C, pH 6.5	500:1	Continuous
Bromelain	8 U @ 37°C, pH 4.6	5,000:1	Banding & Continuous
Protease VIII	12 U @ 37°C, pH 8.5	7,000:1	Banding
Papain	17 U @ 37°C, pH 6.5	10,000:1	Continuous
Protease X	42 U @ 37°C, pH 8.5	20,000:1	Banding

25 Example 12: Antibodies to recombinant human collagen type I directed against different recombinant gelatins

Human recombinant type I collagen produced in the yeast *Pichia pastoris* was tested for its potential allergic reaction as a contact sensitizer on guinea pig, known as Maximization Study. After the duration of the study, the sera were collected to investigate the immunogenicity of recombinant human type I collagen in guinea pig. One gram of rhC I was immersed in 10 ml of either 0.9% Sodium Chloride Injection (SCI) or sesame oil, and incubated for 72 hours at 37°C. The extract was then centrifuge at 3000 rpm for 15 minutes and the supernatant collected for dosing.

Hartley pigs were exposed to the test article and control solution by an induction phase. This phase involved three pairs of intradermal (ID) injections on clipped areas. The first pair of ID

35

5 injections (cranial) consisted of an emulsion of Freud's Complete Adjuvant (FCA) in an equal volume of SCI. The second pair of ID injections (middle) consisted of the test extract (recombinant human type I collagen). The third pair (caudal) consisted of an emulsion of the test extract article and equal volume of FCA. Positive and negative control animals were treated in a similar manner as the test animals, except that the test extract was not included in  
10 the second and third pair of injections.

On the sixth day after ID injections, the test sites were evaluated for evidence of irritation. The test sites were then pretreated with 10% SLS in petroleum and massaged into the skin using a glass rod, and then left uncovered for 24 hours. On the seventh day, a topical  
15 application was administered on the shaved areas of each test animals with 4.25 cm diameter disk of Whatman #3 filter paper soaked with 0.4 ml the test article extract. Thirteen days after the topical induction application, the animals were challenged. An area on the right side of each animal was clipped. On the next day, Hill Top chambers containing 0.3 ml of test extract, vehicle control extract, or positive control solutions were applied to clipped areas and  
20 remained on the animals for 24 hours. The dosing sites were scored for erythema and edema 24, 48, and 72 hours after removal of the chambers.

After 72 hours, the blood was collected and allowed to clot, then centrifuged at 2800 rpm for 15 minutes. The serum was removed from each tube and serum samples were stored at -70°C  
25 until use.

Sera from the immunized Guinea pigs were then analyzed for the presence of antibodies directed against recombinant human collagen type I (rhCI), recombinant human collagen type III (rhCIII), vitron (vitr), and various fragments of recombinant human gelatins of the  
30 present invention, including 6 kDa (SEQ ID NO:18), 10 kDa (SEQ ID NO:19), 18 kDa (SEQ ID NO:20), 33 kDa (SEQ ID NO:27), 50 kDa (SEQ ID NO:22), and 65 kDa (SEQ ID NO:33) fragments. (See Table 2 and Example 1.) Recombinant collagen and recombinant gelatin were electrophoresed on 8% Tris-Glycine or 10-20% Tricine SDS-PAGE gels. Western blot analysis was performed using anti-serum from each of the Guinea pigs used in the study.  
35 Figure 14 shows that recombinant human type I collagen-specific antibodies were present in the sera of Guinea pigs immunized with recombinant human type I collagen. No antibody reactivity to any of the recombinant gelatins analyzed by Western blot analysis was observed in any of the sera of examined. Figure 14 shows Western blot results using the antisera from

5 one Guinea pig in the study. The sera from at least 4 different Guinea pigs were analyzed, each of which showed identical results to that disclosed in Figure 14.

It was desirable to elucidate possible epitopes of the type I collagen responsible for the antigenic response observed following injection of rhcI into Guinea pigs. Recombinant  
10 human collagen type I was separated into its  $\alpha 1(I)$  and  $\alpha 2(I)$  components following denaturation and column chromatography. Cyanogen bromide (CNBr) cleavage of the  $\alpha 1(I)$  chain of recombinant type I collagen and the  $\alpha 2(I)$  chain of recombinant type I collagen was performed as described in Bornstein and Piez (1966) Biochemistry 5:3460. The intact  $\alpha$  chains and the resulting peptide fragments were separated by SDS-PAGE and analyzed by  
15 Western blot analysis for reactivity to the Guinea pig sera described above. Figure 15A shows a coomassie-stained SDS-PAGE of intact and CNBr-cleaved  $\alpha 1(I)$  and  $\alpha 2(I)$  chains of recombinant human type I collagen. Western blot analysis showed that the Guinea pig antisera reactive to rhcI were directed against the  $\alpha 2$  chain of type I collagen and specific CNBr fragments thereof. No reactivity against the  $\alpha 1$  chain of type I collagen was detected.  
20 (Figure 15B.)

The Western blot analyses described above examined the reactivity of the Guinea pig sera to recombinant human type I collagen, CNBr fragments, and recombinant human gelatins by virtue of electrophoretic separation on SDS-PAGE. To examine the reactivity of the Guinea  
25 pig antisera to these polypeptides under non-denatured conditions, a direct ELISA analysis was performed. (Figure 16.) The data showed that the Guinea pig antisera recognized the native conformation of rhcI. None of the recombinant gelatins of the present invention reacted with the Guinea pig antisera by ELISA, regardless of whether the gelatin fragments were presented before or after thermal denaturation. The rhcI was even more reactive in the  
30 ELISA if heat-denatured prior to analysis (data not shown). This indicated the polyclonal antibodies in the sera recognized primarily sequenced epitopes, rather than helical structures. Together, these results indicated that the concerns associated with having an antigenic site(s) present on human collagen type I, specifically to the  $\alpha 2$  chain as shown in the current example, could be avoided by the methods of the present invention. The present invention  
35 thus provides methods for generating recombinant gelatins lacking antigenic sites, which would be useful for specific applications in which gelatin of low antigenicity is desired.

- 5 Various modifications and variations of the described methods and systems of the invention will be apparent to those skilled in the art without departing from the spirit and scope of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Various modifications of the described modes for
- 10 carrying out the invention which are obvious to those skilled in the present art and related fields are intended to be within the scope of the following claims. All references cited herein are incorporated by reference herein in their entirety.

5

## CLAIMS

What is claimed is:

10

1. A vaccine formulation comprising recombinant gelatin.

2. A vaccine formulation comprising recombinant human gelatin.

15

3. The vaccine formulation of claim 1, wherein the recombinant gelatin is non-immunogenic.

4. The vaccine formulation of claim 1, wherein the recombinant gelatin confers stability at ambient temperatures.

20

5. The vaccine formulation of claim 1, wherein the recombinant gelatin is derived from non-native collagen sequence.

25

6. The vaccine formulation of claim 1, wherein the recombinant gelatin has a molecular weight range selected from the group consisting of about 0 to 50 kDa, about 10 to 30 kDa, about 30 to 50 kDa, about 10 to 70 kDa, about 50 kDa to 70 kDa, about 50 to 100 kDa, about 100 to 150 kDa, about 150 to 200 kDa, about 200 to 250 kDa, about 250 to 300 kDa, and about 300 to 350 kDa.

30

7. The vaccine formulation of claim 1, wherein the recombinant gelatin has a molecular weight selected from the group consisting of about 1 kDa, about 5 kDa, about 8 kDa, about 9 kDa, about 14 kDa, about 16 kDa, about 22 kDa, about 23 kDa, about 44 kDa, and about 65 kDa.

35

8. The vaccine formulation of claim 1, wherein the recombinant gelatin is derived from one collagen free of any other type of collagen.

9. The vaccine formulation of claim 1, wherein the recombinant gelatin is produced by processing of recombinant collagen.

- 5 10. The vaccine formulation of claim 1, wherein the recombinant gelatin is produced directly from an altered collagen construct.
11. The vaccine formulation of claim 1, wherein the recombinant gelatin comprises a sequence selected from the group consisting of SEQ ID NOs:15 through 25, and 30, 31, and 33.
- 10 12. The vaccine formulation of claim 1, wherein the vaccine formulation is suitable for injectable delivery.
- 15 13. The vaccine formulation of claim 1, wherein the vaccine formulation is suitable for nasal delivery.
14. The vaccine formulation of claim 1, wherein the vaccine formulation is suitable for oral delivery.
- 20 15. The vaccine formulation of claim 1, wherein the vaccine formulation is suitable for transdermal delivery.
- 25 16. The vaccine formulation of claim 1, wherein the vaccine formulation is suitable for deep lung delivery.
17. The vaccine formulation of claim 1, wherein the vaccine formulation is liquid.
18. The vaccine formulation of claim 1, wherein the vaccine formulation is dry.
- 30 19. The vaccine formulation of claim 1, wherein the vaccine formulation is powdered.
20. The vaccine formulation of claim 1, wherein the vaccine formulation is a spray.
- 35 21. The vaccine formulation of claim 1, wherein the vaccine formulation is an inhalant.
22. The vaccine formulation of claim 1, wherein the vaccine formulation comprises a live vaccine.

- 5     23.     The vaccine formulation of claim 1, wherein the vaccine formulation comprises an inactivated vaccine.
24.     The vaccine formulation of claim 1, wherein the vaccine formulation comprises a subunit vaccine.
- 10     25.     The vaccine formulation of claim 1, wherein the vaccine formulation comprises a single dosage.
26.     The vaccine formulation of claim 1, wherein the vaccine formulation comprises a multiple dosage.
- 15     27.     The vaccine formulation of claim 1, wherein the vaccine formulation comprises a conjugate vaccine.
28.     The vaccine formulation of claim 1, wherein the vaccine formulation comprises a nucleic acid vaccine.
29.     The vaccine formulation of claim 28, wherein the nucleic acid vaccine is a DNA vaccine.
- 25     30.     The vaccine formulation of claim 1, wherein the vaccine formulation is a combined vaccine.
31.     The vaccine formulation of claim 1, wherein the vaccine formulation comprises an acellular vaccine.
- 30     32.     The vaccine formulation of claim 1, wherein the vaccine formulation comprises a vaccine formulated for the prevention of a disease selected from the group consisting of vaccinia virus (small pox), polio virus (Salk and Sabin), mumps, measles, rubella, diphtheria, tetanus, Varicella-Zoster (chicken pox/shingles), pertussis (whooping cough), Bacille Calmette-Guerin (BCG, tuberculosis), haemophilus influenzae meningitis, rabies, cholera, Japanese encephalitis virus, salmonella typhi, shigella, hepatitis A, hepatitis B, adenovirus, yellow fever, foot-and-mouth disease, herpes simplex virus,
- 35

- 5           respiratory syncytial virus, rotavirus, Dengue, West Nile virus, Turkey herpes virus  
(Marek's Disease), influenza, and anthrax.
33.       The vaccine formulation of claim 1, wherein the recombinant gelatin has an endotoxin  
level of below 1.000 EU/mg.
- 10       34.       The vaccine formulation of claim 1, wherein the recombinant gelatin has an endotoxin  
level of below 0.500 EU/mg.
35.       The vaccine formulation of claim 1, wherein the recombinant gelatin has an endotoxin  
15       level of below 0.050 EU/mg.
36.       The vaccine formulation of claim 1, wherein the recombinant gelatin has an endotoxin  
level of below 0.005 EU/mg.
- 20       37.       The vaccine formulation of claim 1, wherein the recombinant gelatin is proteolytically  
stable.
38.       A vaccine stabilizer comprising recombinant gelatin.
- 25       39.       A method of producing a vaccine comprising recombinant gelatin, the method  
comprising:  
          providing a vaccine;  
30       providing recombinant gelatin; and  
          combining the vaccine and the recombinant gelatin.
40.       A method of inducing an immune response in a subject, the method comprising  
35       recombinant gelatin, the method comprising administering the vaccine comprising  
recombinant gelatin to the subject.



- 5     41.     A kit, the kit comprising:
- (i)     a vaccine comprising recombinant gelatin; and
- (ii)    a delivery device for delivery of the vaccine.
- 10
42.     The vaccination kit of claim 41, wherein the delivery device is a device for injectable delivery.
43.     The vaccination kit of claim 41, wherein the delivery device is a device for nasal delivery.
- 15
44.     The vaccination kit of claim 41, wherein the delivery device is a device for mucosal delivery.
45.     The vaccination kit of claim 41, wherein the delivery device is a device for aerosol delivery.
- 20

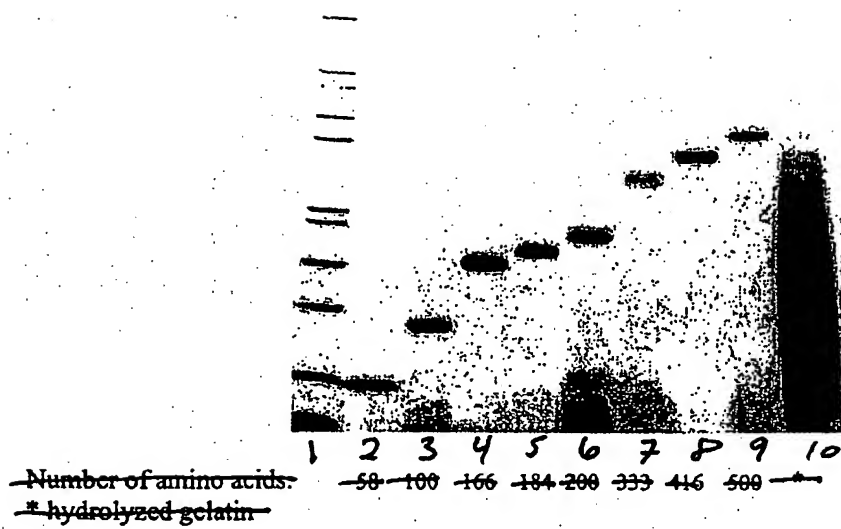


FIGURE 1

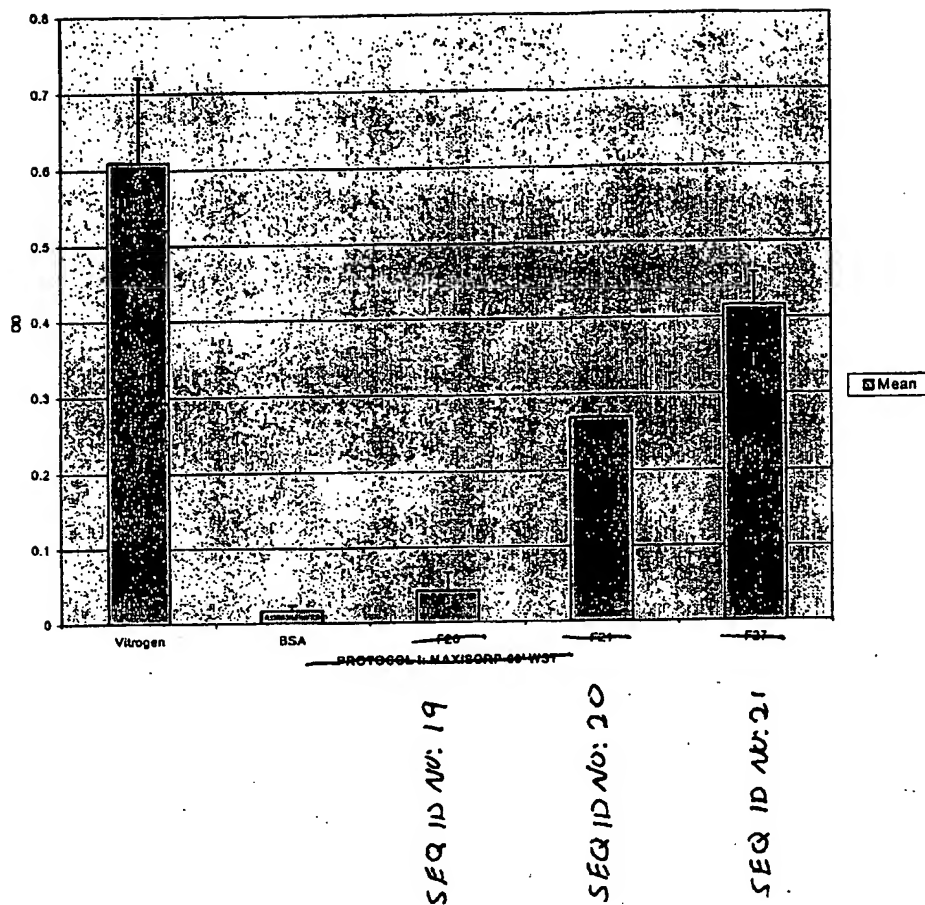


FIGURE 2A

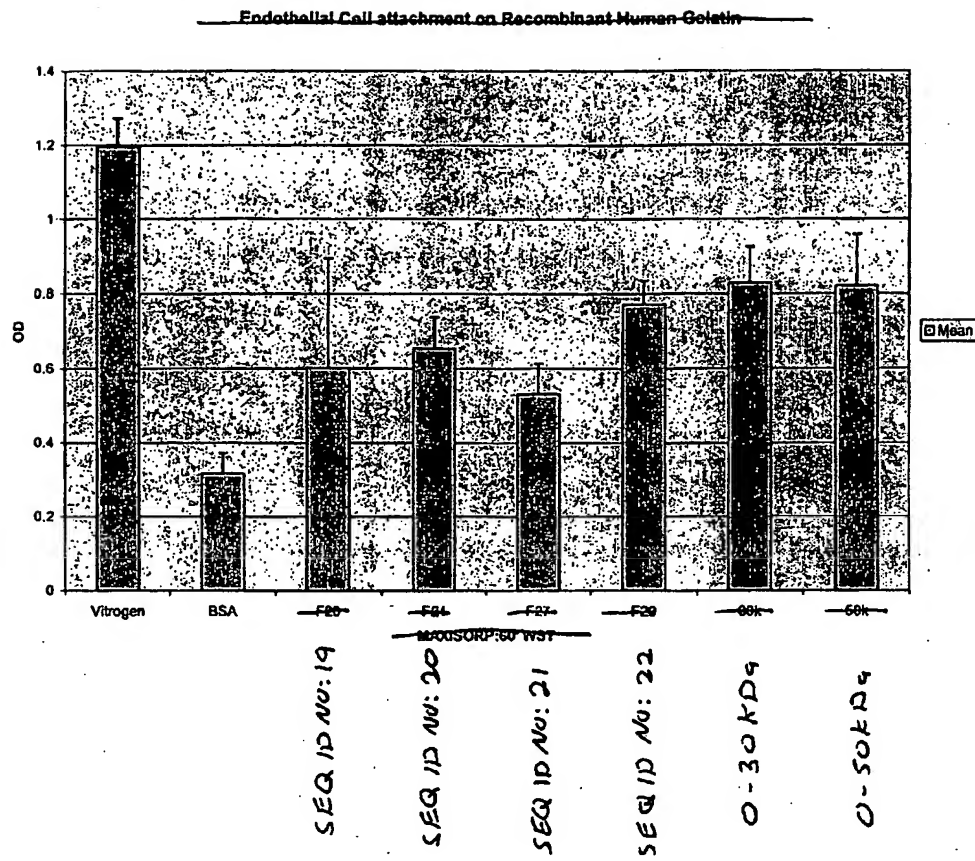


FIGURE 2B

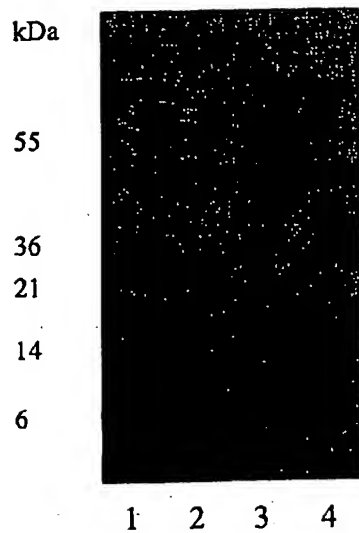


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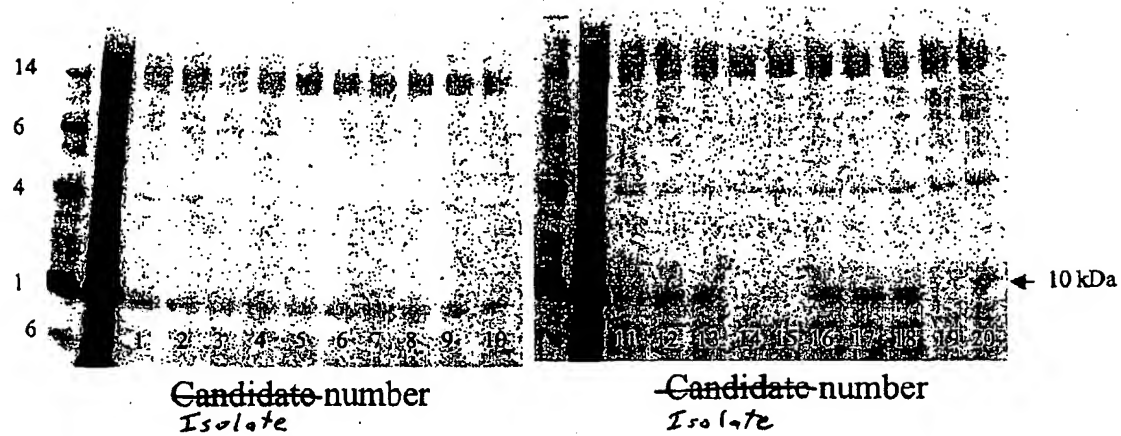


FIGURE 4

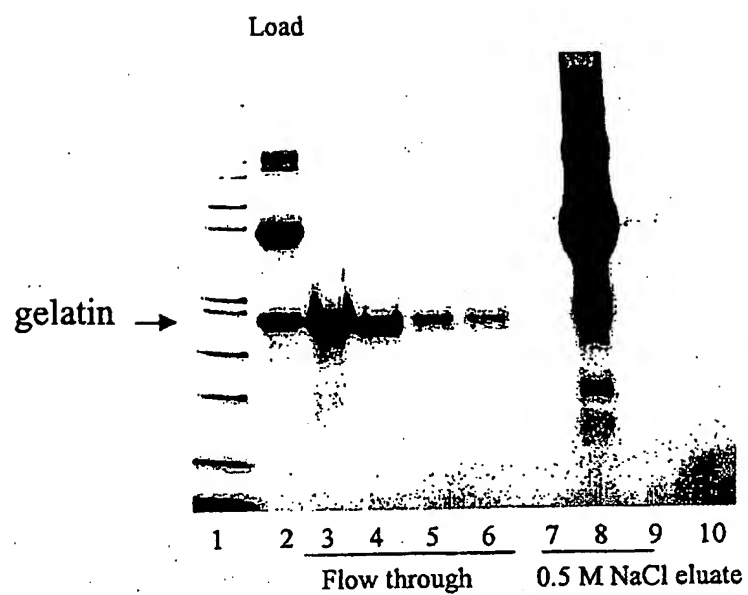


FIGURE 5

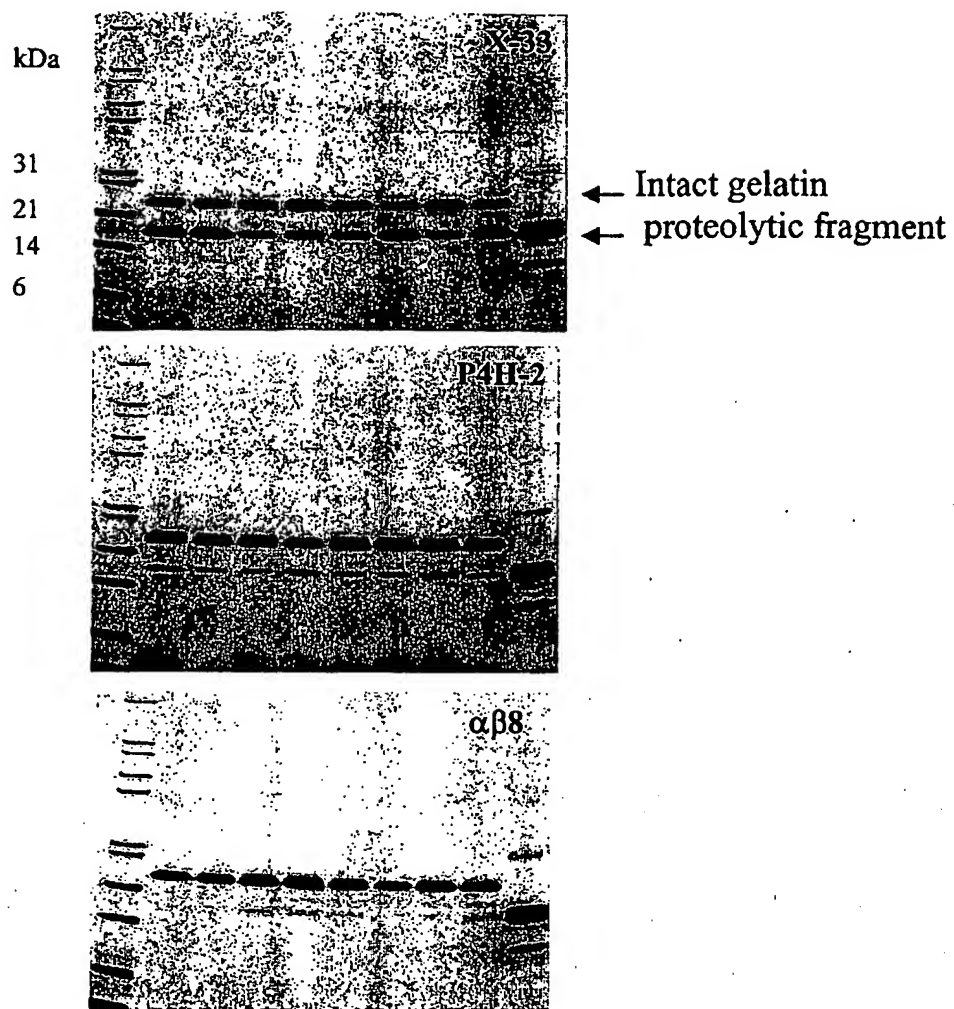


Figure 6



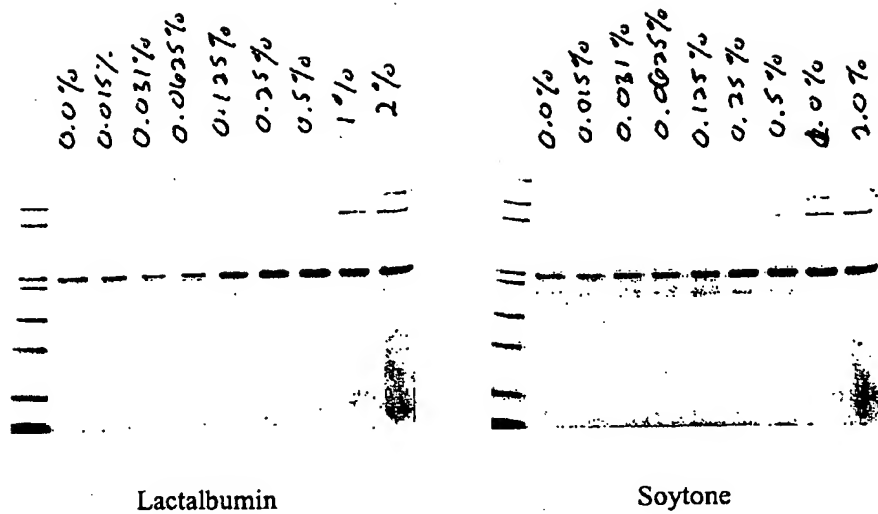


FIGURE 7



FIGURE 8

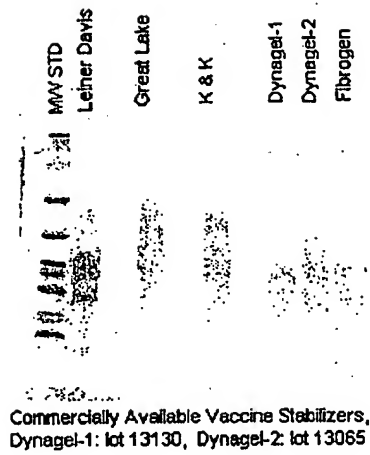
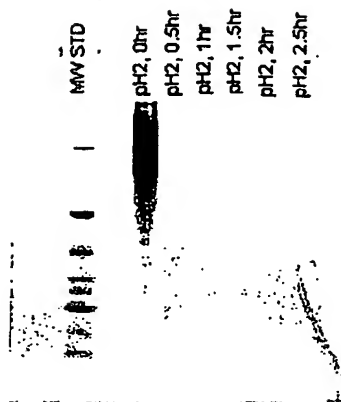
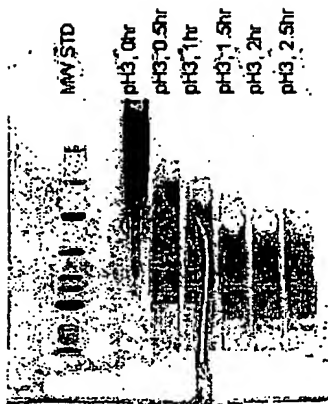


FIGURE 9



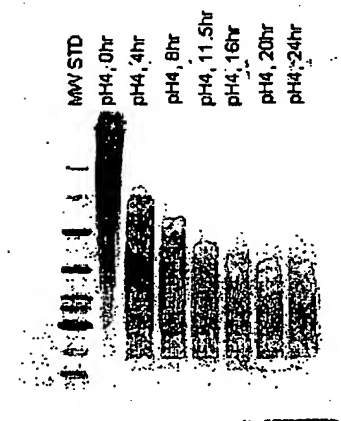
Hydrolysis of Sigma Gelatin @ 120C

FIGURE 10A



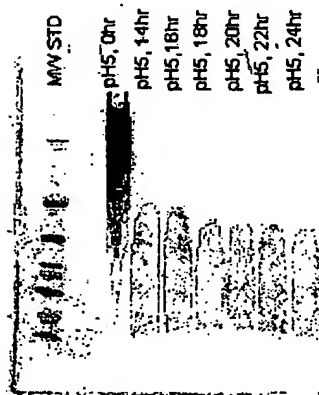
Hydrolysis of Sigma Gelatin @ 120C

FIGURE 10B



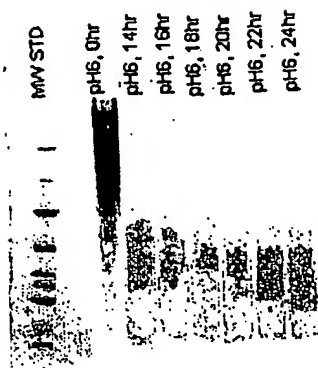
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FIGURE 10C



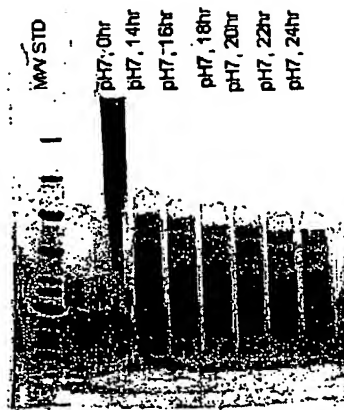
Hydrolysis of Sigma Gelatin @ 120C

FIGURE 10D



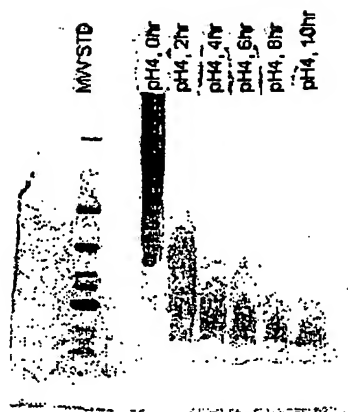
Hydrolysis of Sigma Gelatin @ 120C

FIGURE 10E



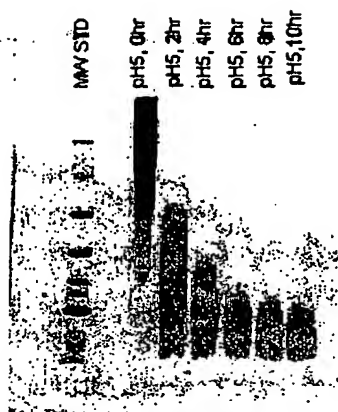
Hydrolysis of Sigma Gelatin @ 120C

FIGURE 10F



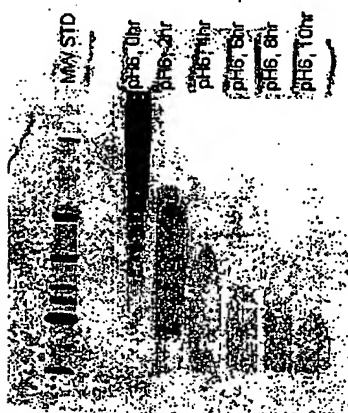
Hydrolysis of Sigma Gelatin @ 150C

FIGURE 11A



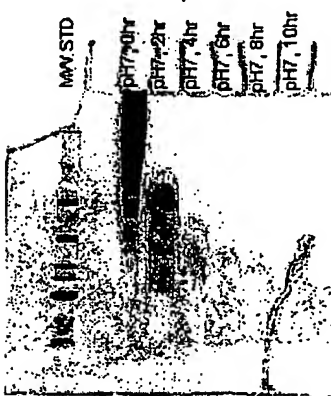
Hydrolysis of Sigma Gelatin @ 150C

FIGURE 11B



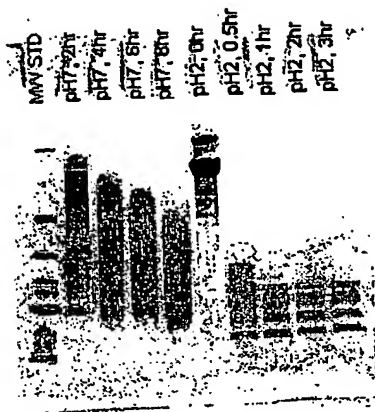
Hydrolysis of Sigma Gelatin @ 150C

FIGURE 11C



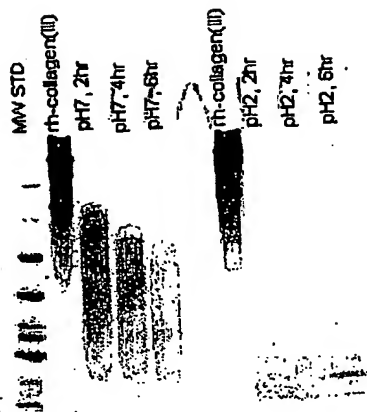
Hydrolysis of Sigma Gelatin @ 150C

FIGURE 11D



Hydrolysis of rh-collagen (I) @ 120C

FIGURE 12A



Hydrolysis of rh-collagen(II) @120C

FIGURE 12B

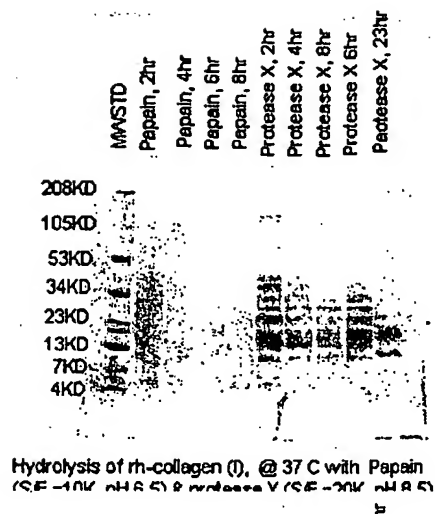


FIGURE 13



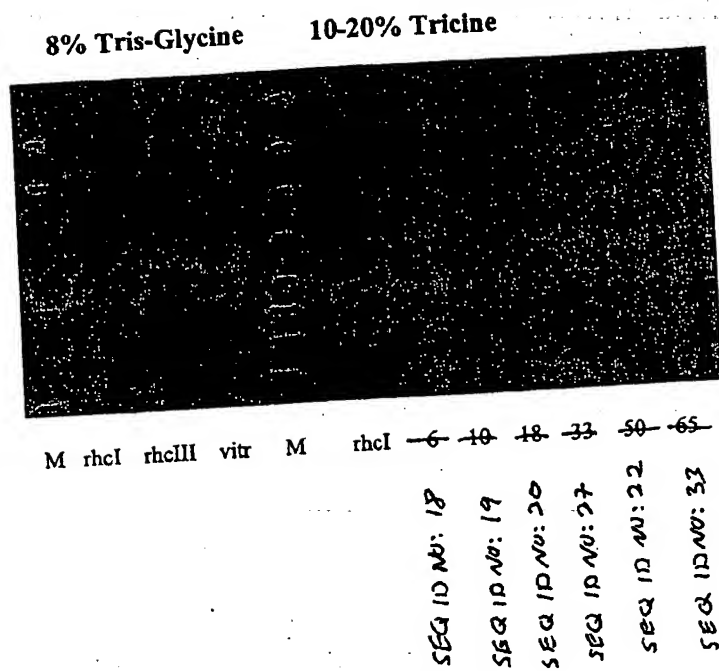
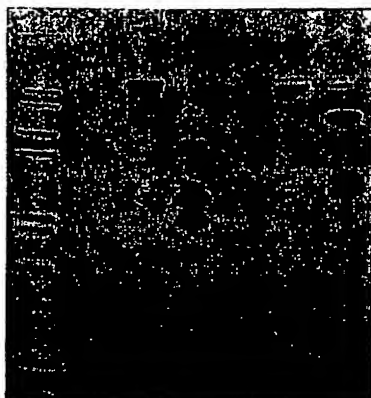


FIGURE 14



$\alpha 1(I)$   $\alpha 1(I)$   $\alpha 2(I)$   $\alpha 2(I)$   
+CNBr +CNBr

FIGURE 15A

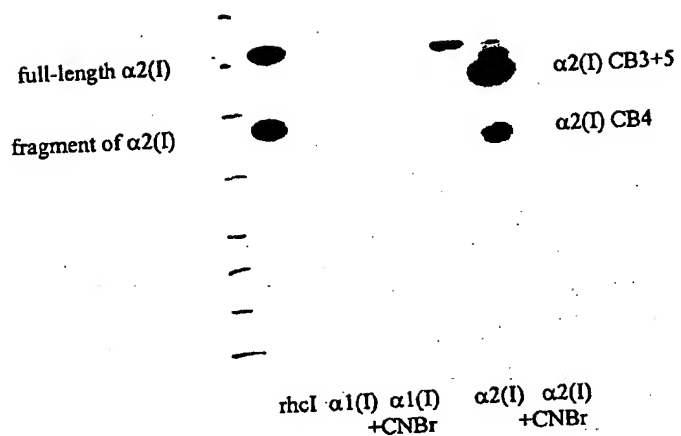


FIGURE 15B

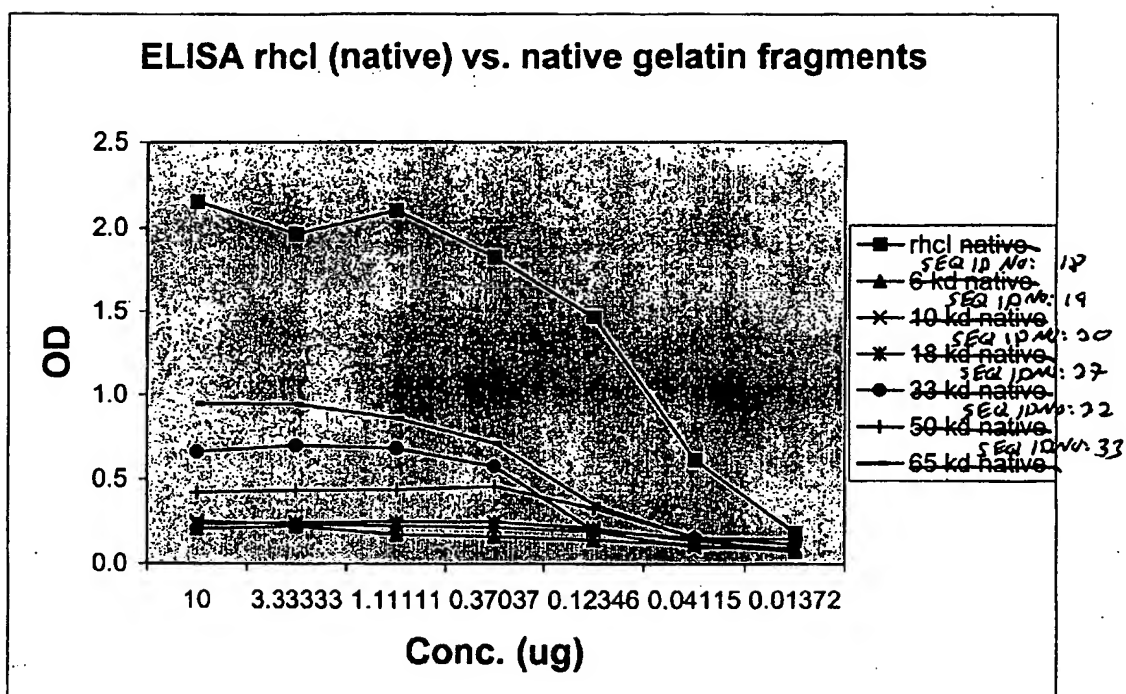


FIGURE 16

## SEQUENCE LISTING

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Glu Arg Gly Pro Pro Gly Pro Gln Gly Ala Arg Gly Leu Pro Gly Thr  
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Ala Pro Gly Pro Gln Gly Phe Gln Gly Pro Pro Gly Glu Pro Gly Glu  
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Pro Gly Ala Ser Gly Pro Met Gly Pro Arg Gly Pro Pro Gly Pro Pro  
35 40 45

Gly Lys Asn Gly Asp Asp Gly Glu Ala Gly Lys Pro Gly Arg Pro Gly  
50 55 60

Glu Arg Gly Pro Pro Gly Pro Gln Gly Ala Arg Gly Leu Pro Gly Thr  
65 70 75 80



Ala Gly Leu Pro y Met Lys Gly His Arg Gly Phe Ser Gly Leu Asp  
 5 90 95  
 Gly Ala Lys Gly Asp Ala Gly Pro Ala Gly Pro Lys Gly Glu Pro Gly  
 100 105 110  
 Ser Pro Gly Glu Asn Gly Ala Pro Gly Gln Met Gly Pro Arg Gly Leu  
 115 120 125  
 Pro Gly Glu Arg Gly Arg Pro Gly Ala Pro Gly Pro Ala Gly Ala Arg  
 130 135 140  
 Gly Asn Asp Gly Ala Thr Gly Ala Ala Gly Pro Pro Gly Pro Thr Gly  
 145 150 155 160  
 Pro Ala Gly Pro Pro Gly Phe Pro Gly Ala Val Gly Ala Lys Gly Glu  
 165 170 175  
 Ala Gly Pro Gln Gly Pro Arg Gly Ser Glu Gly Pro Gln Gly Val Arg  
 180 185 190  
 Gly Glu Pro Gly Pro Pro Gly Pro Ala Gly Ala Ala Gly Pro Ala Gly  
 195 200 205  
 Asn Pro Gly Ala Asp Gly Gln Pro Gly Ala Lys Gly Ala Asn Gly Ala  
 210 215 220  
 Pro Gly Ile Ala Gly Ala Pro Gly Phe Pro Gly Ala Arg Gly Pro Ser  
 225 230 235 240  
 Gly Pro Gln Gly Pro Gly Gly Pro Pro Gly Pro Lys Gly Asn Ser Gly  
 245 250 255  
 Glu Pro Gly Ala Pro Gly Ser Lys Gly Asp Thr Gly Ala Lys Gly Glu  
 260 265 270  
 Pro Gly Pro Val Gly Val Gln Gly Pro Pro Gly Pro Ala Gly Glu Glu  
 275 280 285  
 Gly Lys Arg Gly Ala Arg Gly Glu Pro Gly Pro Thr Gly Leu Pro Gly  
 290 295 300  
 Pro Pro Gly Glu Arg Gly Gly Pro Gly Ser Arg Gly Phe Pro Gly Ala  
 305 310 315 320  
 Asp Gly Val Ala Gly Pro Lys Gly Pro Ala Gly Glu Arg Gly Ser Pro  
 325 330 335  
 Gly Pro Ala Gly Pro Lys Gly Ser Pro Gly Glu Ala Gly Arg Pro Gly  
 340 345 350  
 Glu Ala Gly Leu Pro Gly Ala Lys Gly Leu Thr Gly Ser Pro Gly Ser  
 355 360 365  
 Pro Gly Pro Asp Gly Lys Thr Gly Pro Pro Gly Pro Ala Gly Gln Asp  
 370 375 380  
 Gly Arg Pro Gly Pro Pro Gly Pro Pro Gly Ala Arg Gly Gln Ala Gly  
 385 390 395 400

Val Met Gly Phe Gly Pro Lys Gly Ala Ala Gly Glu Pro Gly Lys  
 405 410 415

Ala Gly Glu Arg Gly Val Pro Gly Pro Pro Gly Ala Val Gly Pro Ala  
 420 425 430

Gly Lys Asp Gly Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly  
 435 440 445

Pro Ala Gly Glu Arg Gly Glu Gln Gly Pro Ala Gly Ser Pro Gly Phe  
 450 455 460

Gln Gly Leu Pro Gly Pro Ala Gly Pro Pro Gly Glu Ala Gly Lys Pro  
 465 470 475 480

Gly Glu Gln Gly Val Pro Gly Asp Leu Gly Ala Pro Gly Pro Ser Gly  
 485 490 495

Ala Arg Gly Glu Arg  
 500

<210> 18  
 <211> 59  
 <212> PRT  
 <213> human

<400> 18  
 Glu Ala Gly Leu Pro Gly Ala Lys Gly Leu Thr Gly Ser Pro Gly Ser  
 1 5 10 15

Pro Gly Pro Asp Gly Lys Thr Gly Pro Pro Gly Pro Ala Gly Gln Asp  
 20 25 30

Gly Arg Pro Gly Pro Pro Gly Pro Pro Gly Ala Arg Gly Gln Ala Gly  
 35 40 45

Val Met Gly Phe Pro Gly Pro Lys Gly Ala Ala  
 50 55

<210> 19  
 <211> 101  
 <212> PRT  
 <213> human

<400> 19  
 Glu Ala Gly Leu Pro Gly Ala Lys Gly Leu Thr Gly Ser Pro Gly Ser  
 1 5 10 15

Pro Gly Pro Asp Gly Lys Thr Gly Pro Pro Gly Pro Ala Gly Gln Asp  
 20 25 30

Gly Arg Pro Gly Pro Pro Gly Pro Pro Gly Ala Arg Gly Gln Ala Gly  
 35 40 45

Val Met Gly Phe Pro Gly Pro Lys Gly Ala Ala Gly Glu Pro Gly Lys  
 50 55 60

Ala Gly Glu Arg y Val Pro Gly Pro Pro Gly Ala Val Gly Pro Ala  
65 70 75 80

Gly Lys Asp Gly Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly  
85 90 95

Pro Ala Gly Glu Arg  
100

<210> 20  
<211> 185  
<212> PRT  
<213> human

<400> 20  
Glu Ala Gly Leu Pro Gly Ala Lys Gly Leu Thr Gly Ser Pro Gly Ser  
1 5 10 15

Pro Gly Pro Asp Gly Lys Thr Gly Pro Gly Pro Ala Gly Gln Asp  
20 25 30

Gly Arg Pro Gly Pro Pro Gly Pro Pro Gly Ala Arg Gly Gln Ala Gly  
35 40 45

Val Met Gly Phe Pro Gly Pro Lys Gly Ala Ala Gly Glu Pro Gly Lys  
50 55 60

Ala Gly Glu Arg Gly Val Pro Gly Pro Pro Gly Ala Val Gly Pro Ala  
65 70 75 80

Gly Lys Asp Gly Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly  
85 90 95

Pro Ala Gly Glu Arg Gly Glu Gln Gly Pro Ala Gly Ser Pro Gly Phe  
100 105 110

Gln Gly Leu Pro Gly Pro Ala Gly Pro Pro Gly Glu Ala Gly Lys Pro  
115 120 125

Gly Glu Gln Gly Val Pro Gly Asp Leu Gly Ala Pro Gly Pro Ser Gly  
130 135 140

Ala Arg Gly Glu Arg Gly Phe Pro Gly Glu Arg Gly Val Gln Gly Pro  
145 150 155 160

Pro Gly Pro Ala Gly Pro Arg Gly Ala Asn Gly Ala Pro Gly Asn Asp  
165 170 175

Gly Ala Lys Gly Asp Ala Gly Ala Pro  
180 185

<210> 21  
<211> 251  
<212> PRT  
<213> human

<400> 21

Glu Ala Gly Leu 1 5 Gly Ala Lys Gly Leu Thr Gly Ser Pro Gly Ser 10 15  
 Pro Gly Pro Asp Gly Lys Thr Gly Pro Pro Gly Pro Ala Gly Gln Asp 20 25 30  
 Gly Arg Pro Gly Pro Pro Gly Pro Pro Gly Ala Arg Gly Gln Ala Gly 35 40 45  
 Val Met Gly Phe Pro Gly Pro Lys Gly Ala Ala Gly Glu Pro Gly Lys 50 55 60  
 Ala Gly Glu Arg Gly Val Pro Gly Pro Pro Gly Ala Val Gly Pro Ala 65 70 75 80  
 Gly Lys Asp Gly Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly 85 90 95  
 Pro Ala Gly Glu Arg Gly Glu Gln Gly Pro Ala Gly Ser Pro Gly Phe 100 105 110  
 Gln Gly Leu Pro Gly Pro Ala Gly Pro Pro Gly Glu Ala Gly Lys Pro 115 120 125  
 Gly Glu Gln Gly Val Pro Gly Asp Leu Gly Ala Pro Gly Pro Ser Gly 130 135 140  
 Ala Arg Gly Glu Arg Gly Phe Pro Gly Glu Arg Gly Val Gln Gly Pro 145 150 155 160  
 Pro Gly Pro Ala Gly Pro Arg Gly Ala Asn Gly Ala Pro Gly Asn Asp 165 170 175  
 Gly Ala Lys Gly Asp Ala Gly Ala Pro Gly Ala Pro Gly Ser Gln Gly 180 185 190  
 Ala Pro Gly Leu Gln Gly Met Pro Gly Glu Arg Gly Ala Ala Gly Leu 195 200 205  
 Pro Gly Pro Lys Gly Asp Arg Gly Asp Ala Gly Pro Lys Gly Ala Asp 210 215 220  
 Gly Ser Pro Gly Lys Asp Gly Val Arg Gly Leu Thr Gly Pro Ile Gly 225 230 235 240  
 Pro Pro Gly Pro Ala Gly Ala Pro Gly Asp Lys 245 250

<210> 22  
 <211> 500  
 <212> PRT  
 <213> human

<400> 22  
 Glu Ala Gly Leu Pro Gly Ala Lys Gly Leu Thr Gly Ser Pro Gly Ser  
 1 5 10 15

Pro Gly Pro Asp 20 y Lys Thr Gly Pro Pro Gly Pro Ala Gly Gln Asp 30  
 Gly Arg Pro Gly Pro Pro Gly Pro Pro Gly Ala Arg Gly Gln Ala Gly 45  
 Val Met Gly Phe Pro Gly Pro Lys Gly Ala Ala Gly Glu Pro Gly Lys 60  
 Ala Gly Glu Arg Gly Val Pro Gly Pro Pro Gly Ala Val Gly Pro Ala 80  
 Gly Lys Asp Gly Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly 95  
 Pro Ala Gly Glu Arg Gly Glu Gln Gly Pro Ala Gly Ser Pro Gly Phe 110  
 Gln Gly Leu Pro Gly Pro Ala Gly Pro Pro Gly Glu Ala Gly Lys Pro 125  
 Gly Glu Gln Gly Val Pro Gly Asp Leu Gly Ala Pro Gly Pro Ser Gly 140  
 Ala Arg Gly Glu Arg Gly Phe Pro Gly Glu Arg Gly Val Gln Gly Pro 160  
 Pro Gly Pro Ala Gly Pro Arg Gly Ala Asn Gly Ala Pro Gly Asn Asp 175  
 Gly Ala Lys Gly Asp Ala Gly Ala Pro Gly Ala Pro Gly Ser Gln Gly 190  
 Ala Pro Gly Leu Gln Gly Met Pro Gly Glu Arg Gly Ala Ala Gly Leu 205  
 Pro Gly Pro Lys Gly Asp Arg Gly Asp Ala Gly Pro Lys Gly Ala Asp 220  
 Gly Ser Pro Gly Lys Asp Gly Val Arg Gly Leu Thr Gly Pro Ile Gly 240  
 Pro Pro Gly Pro Ala Gly Ala Pro Gly Asp Lys Gly Glu Ser Gly Pro 255  
 Ser Gly Pro Ala Gly Pro Thr Gly Ala Arg Gly Ala Pro Gly Asp Arg 270  
 Gly Glu Pro Gly Pro Pro Gly Pro Ala Gly Phe Ala Gly Pro Pro Gly 285  
 Ala Asp Gly Gln Pro Gly Ala Lys Gly Glu Pro Gly Asp Ala Gly Ala 300  
 Lys Gly Asp Ala Gly Pro Pro Gly Pro Ala Gly Pro Ala Gly Pro Pro 320  
 Gly Pro Ile Gly Asn Val Gly Ala Pro Gly Ala Lys Gly Ala Arg Gly 335

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<210> 23
<211> 91
<212> PRT
<213> human
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Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly Pro Ala Gly Glu  
 1 5 10 15  
 Arg Gly Glu Gln Gly Pro Ala Gly Ser Pro Gly Phe Gln Gly Leu Pro  
 20 25 30  
 Gly Pro Ala Gly Pro Pro Gly Glu Ala Gly Lys Pro Gly Glu Gln Gly  
 35 40 45  
 Val Pro Gly Asp Leu Gly Ala Pro Gly Pro Ser Gly Ala Arg Gly Glu  
 50 55 60  
 Arg Gly Phe Pro Gly Glu Arg Gly Val Gln Gly Pro Pro Gly Pro Ala  
 65 70 75 80  
 Gly Pro Arg Gly Ala Asn Gly Ala Pro Gly Asn  
 85 90

<210> 24  
 <211> 167  
 <212> PRT  
 <213> human

<400> 24  
 Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly Pro Ala Gly Glu  
 1 5 10 15  
 Arg Gly Glu Gln Gly Pro Ala Gly Ser Pro Gly Phe Gln Gly Leu Pro  
 20 25 30  
 Gly Pro Ala Gly Pro Pro Gly Glu Ala Gly Lys Pro Gly Glu Gln Gly  
 35 40 45  
 Val Pro Gly Asp Leu Gly Ala Pro Gly Pro Ser Gly Ala Arg Gly Glu  
 50 55 60  
 Arg Gly Phe Pro Gly Glu Arg Gly Val Gln Gly Pro Pro Gly Pro Ala  
 65 70 75 80  
 Gly Pro Arg Gly Ala Asn Gly Ala Pro Gly Asn Asp Gly Ala Lys Gly  
 85 90 95  
 Asp Ala Gly Ala Pro Gly Ala Pro Gly Ser Gln Gly Ala Pro Gly Leu  
 100 105 110  
 Gln Gly Met Pro Gly Glu Arg Gly Ala Ala Gly Leu Pro Gly Pro Lys  
 115 120 125  
 Gly Asp Arg Gly Asp Ala Gly Pro Lys Gly Ala Asp Gly Ser Pro Gly  
 130 135 140  
 Lys Asp Gly Val Arg Gly Leu Thr Gly Pro Ile Gly Pro Pro Gly Pro  
 145 150 155 160  
 Ala Gly Ala Pro Gly Asp Lys  
 165

<210> 25  
 <211> 416  
 <212> PRT  
 <213> human

<400> 25  
 Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly Pro Ala Gly Glu  
 1 5 10 15  
 Arg Gly Glu Gln Gly Pro Ala Gly Ser Pro Gly Phe Gln Gly Leu Pro  
 20 25 30  
 Gly Pro Ala Gly Pro Pro Gly Glu Ala Gly Lys Pro Gly Glu Gln Gly  
 35 40 45  
 Val Pro Gly Asp Leu Gly Ala Pro Gly Pro Ser Gly Ala Arg Gly Glu  
 50 55 60

Arg Gly Phe Pro y Glu Arg Gly Val Gln Gly Pro Pro Gly Pro Ala  
 65 70 75 80  
 Gly Pro Arg Gly Ala Asn Gly Ala Pro Gly Asn Asp Gly Ala Lys Gly  
 85 90 95  
 Asp Ala Gly Ala Pro Gly Ala Pro Gly Ser Gln Gly Ala Pro Gly Leu  
 100 105 110  
 Gln Gly Met Pro Gly Glu Arg Gly Ala Ala Gly Leu Pro Gly Pro Lys  
 115 120 125  
 Gly Asp Arg Gly Asp Ala Gly Pro Lys Gly Ala Asp Gly Ser Pro Gly  
 130 135 140  
 Lys Asp Gly Val Arg Gly Leu Thr Gly Pro Ile Gly Pro Pro Gly Pro  
 145 150 155 160  
 Ala Gly Ala Pro Gly Asp Lys Gly Glu Ser Gly Pro Ser Gly Pro Ala  
 165 170 175  
 Gly Pro Thr Gly Ala Arg Gly Ala Pro Gly Asp Arg Gly Glu Pro Gly  
 180 185 190  
 Pro Pro Gly Pro Ala Gly Phe Ala Gly Pro Pro Gly Ala Asp Gly Gln  
 195 200 205  
 Pro Gly Ala Lys Gly Glu Pro Gly Asp Ala Gly Ala Lys Gly Asp Ala  
 210 215 220  
 Gly Pro Pro Gly Pro Ala Gly Pro Ala Gly Pro Pro Gly Pro Ile Gly  
 225 230 235 240  
 Asn Val Gly Ala Pro Gly Ala Lys Gly Ala Arg Gly Ser Ala Gly Pro  
 245 250 255  
 Pro Gly Ala Thr Gly Phe Pro Gly Ala Ala Gly Arg Val Gly Pro Pro  
 260 265 270  
 Gly Pro Ser Gly Asn Ala Gly Pro Pro Gly Pro Pro Gly Pro Ala Gly  
 275 280 285  
 Lys Glu Gly Gly Lys Gly Pro Arg Gly Glu Thr Gly Pro Ala Gly Arg  
 290 295 300  
 Pro Gly Glu Val Gly Pro Pro Gly Pro Pro Gly Pro Ala Gly Glu Lys  
 305 310 315 320  
 Gly Ser Pro Gly Ala Asp Gly Pro Ala Gly Ala Pro Gly Thr Pro Gly  
 325 330 335  
 Pro Gln Gly Ile Ala Gly Gln Arg Gly Val Val Gly Leu Pro Gly Gln  
 340 345 350  
 Arg Gly Glu Arg Gly Phe Pro Gly Leu Pro Gly Pro Ser Gly Glu Pro  
 355 360 365  
 Gly Lys Gln Gly Pro Ser Gly Ala Ser Gly Glu Arg Gly Pro Pro Gly  
 370 375 380



Pro Met Gly Pro    Gly Leu Ala Gly Pro Pro Gly Glu Ser Gly Arg  
385                            390                            395                            400

Glu Gly Ala Pro Ala Ala Glu Gly Ser Pro Gly Arg Asp Gly Ser Pro  
                          405                            410                            415

<210> 26  
<211> 510  
<212> PRT  
<213> human

<400> 26

Gly Glu Arg Gly Val Gln Gly Pro Pro Gly Pro Ala Gly Pro Arg Gly  
1                            5                            10                            15

Ala Asn Gly Ala Pro Gly Asn Asp Gly Ala Lys Gly Asp Ala Gly Ala  
                          20                            25                            30

Pro Gly Ala Pro Gly Ser Gln Gly Ala Pro Gly Leu Gln Gly Met Pro  
                          35                            40                            45

Gly Glu Arg Gly Ala Ala Gly Leu Pro Gly Pro Lys Gly Asp Arg Gly  
50                            55                            60

Asp Ala Gly Pro Lys Gly Ala Asp Gly Ser Pro Gly Lys Asp Gly Val  
65                            70                            75                            80

Arg Gly Leu Thr Gly Pro Ile Gly Pro Pro Gly Pro Ala Gly Ala Pro  
                          85                            90                            95

Gly Asp Lys Gly Glu Ser Gly Pro Ser Gly Pro Ala Gly Pro Thr Gly  
100                            105                            110

Ala Arg Gly Ala Pro Gly Asp Arg Gly Glu Pro Gly Pro Pro Gly Pro  
115                            120                            125

Ala Gly Phe Ala Gly Pro Pro Gly Ala Asp Gly Gln Pro Gly Ala Lys  
130                            135                            140

Gly Glu Pro Gly Asp Ala Gly Ala Lys Gly Asp Ala Gly Pro Pro Gly  
145                            150                            155                            160

Pro Ala Gly Pro Ala Gly Pro Pro Gly Pro Ile Gly Asn Val Gly Ala  
                          165                            170                            175

Pro Gly Ala Lys Gly Ala Arg Gly Ser Ala Gly Pro Pro Gly Ala Thr  
180                            185                            190

Gly Phe Pro Gly Ala Ala Gly Arg Val Gly Pro Pro Gly Pro Ser Gly  
195                            200                            205

Asn Ala Gly Pro Pro Gly Pro Pro Gly Pro Ala Gly Lys Glu Gly Gly  
210                            215                            220

Lys Gly Pro Arg Gly Glu Thr Gly Pro Ala Gly Arg Pro Gly Glu Val  
225                            230                            235                            240

Gly Pro Pro Gly 245 Pro Gly Pro Ala Gly Glu Lys Gly Ser Pro Gly 255  
 Ala Asp Gly Pro Ala Gly Ala Pro Gly Thr Pro Gly Pro Gln Gly Ile 260 265 270  
 Ala Gly Gln Arg Gly Val Val Gly Leu Pro Gly Gln Arg Gly Glu Arg 275 280 285  
 Gly Phe Pro Gly Leu Pro Gly Pro Ser Gly Glu Pro Gly Lys Gln Gly 290 295 300  
 Pro Ser Gly Ala Ser Gly Glu Arg Gly Pro Pro Gly Pro Met Gly Pro 305 310 315 320  
 Pro Gly Leu Ala Gly Pro Pro Gly Glu Ser Gly Arg Glu Gly Ala Pro 325 330 335  
 Ala Ala Glu Gly Ser Pro Gly Arg Asp Gly Ser Pro Gly Ala Lys Gly 340 345 350  
 Asp Arg Gly Glu Thr Gly Pro Ala Gly Pro Pro Gly Ala Pro Gly Ala 355 360 365  
 Pro Gly Ala Pro Gly Pro Val Gly Pro Ala Gly Lys Ser Gly Asp Arg 370 375 380  
 Gly Glu Thr Gly Pro Ala Gly Pro Ala Gly Pro Val Gly Pro Val Gly 385 390 395 400  
 Ala Arg Gly Pro Ala Gly Pro Gln Gly Pro Arg Gly Asp Lys Gly Glu 405 410 415  
 Thr Gly Glu Gln Gly Asp Arg Gly Ile Lys Gly His Arg Gly Phe Ser 420 425 430  
 Gly Leu Gln Gly Pro Pro Gly Pro Pro Gly Ser Pro Gly Glu Gln Gly 435 440 445  
 Pro Ser Gly Ala Ser Gly Pro Ala Gly Pro Arg Gly Pro Pro Gly Ser 450 455 460  
 Ala Gly Ala Pro Gly Lys Asp Gly Leu Asn Gly Leu Pro Gly Pro Ile 465 470 475 480  
 Gly Pro Pro Gly Pro Arg Gly Arg Thr Gly Asp Ala Gly Pro Val Gly 485 490 495  
 Pro Pro Gly Pro Pro Gly Pro Pro Gly Pro Pro Gly Pro Pro 500 505 510

&lt;210&gt; 27

&lt;211&gt; 333

&lt;212&gt; PRT

&lt;213&gt; human

&lt;400&gt; 27

Gly Ala Lys Gly a Arg Gly Ser Ala Gly Pro Pro Gly Ala Thr Gly  
 1 5 10 15  
 Phe Pro Gly Ala Ala Gly Arg Val Gly Pro Pro Gly Pro Ser Gly Asn  
 20 25 30  
 Ala Gly Pro Pro Gly Pro Pro Gly Pro Ala Gly Lys Glu Gly Gly Lys  
 35 40 45  
 Gly Pro Arg Gly Glu Thr Gly Pro Ala Gly Arg Pro Gly Glu Val Gly  
 50 55 60  
 Pro Pro Gly Pro Pro Gly Pro Ala Gly Glu Lys Gly Ser Pro Gly Ala  
 65 70 75 80  
 Asp Gly Pro Ala Gly Ala Pro Gly Thr Pro Gly Pro Gln Gly Ile Ala  
 85 90 95  
 Gly Gln Arg Gly Val Val Gly Leu Pro Gly Gln Arg Gly Glu Arg Gly  
 100 105 110  
 Phe Pro Gly Leu Pro Gly Pro Ser Gly Glu Pro Gly Lys Gln Gly Pro  
 115 120 125  
 Ser Gly Ala Ser Gly Glu Arg Gly Pro Pro Gly Pro Met Gly Pro Pro  
 130 135 140  
 Gly Leu Ala Gly Pro Pro Gly Glu Ser Gly Arg Glu Gly Ala Pro Ala  
 145 150 155 160  
 Ala Glu Gly Ser Pro Gly Arg Asp Gly Ser Pro Gly Ala Lys Gly Asp  
 165 170 175  
 Arg Gly Glu Thr Gly Pro Ala Gly Pro Pro Gly Ala Pro Gly Ala Pro  
 180 185 190  
 Gly Ala Pro Gly Pro Val Gly Pro Ala Gly Lys Ser Gly Asp Arg Gly  
 195 200 205  
 Glu Thr Gly Pro Ala Gly Pro Ala Gly Pro Val Gly Pro Val Gly Ala  
 210 215 220  
 Arg Gly Pro Ala Gly Pro Gln Gly Pro Arg Gly Asp Lys Gly Glu Thr  
 225 230 235 240  
 Gly Glu Gln Gly Asp Arg Gly Ile Lys Gly His Arg Gly Phe Ser Gly  
 245 250 255  
 Leu Gln Gly Pro Pro Gly Pro Pro Gly Ser Pro Gly Glu Gln Gly Pro  
 260 265 270  
 Ser Gly Ala Ser Gly Pro Ala Gly Pro Arg Gly Pro Pro Gly Ser Ala  
 275 280 285  
 Gly Ala Pro Gly Lys Asp Gly Leu Asn Gly Leu Pro Gly Pro Ile Gly  
 290 295 300  
 Pro Pro Gly Pro Arg Gly Arg Thr Gly Asp Ala Gly Pro Val Gly Pro  
 305 310 315 320

Pro Gly Pro Pro y Pro Pro Gly Pro Pro Gly Pro Pro  
 325 330

<210> 28  
 <211> 200  
 <212> PRT  
 <213> human

<400> 28

Glu Arg Gly Pro Pro Gly Pro Met Gly Pro Pro Gly Leu Ala Gly Pro  
 1 5 10 15

Pro Gly Glu Ser Gly Arg Glu Gly Ala Pro Ala Ala Glu Gly Ser Pro  
 20 25 30

Gly Arg Asp Gly Ser Pro Gly Ala Lys Gly Asp Arg Gly Glu Thr Gly  
 35 40 45

Pro Ala Gly Pro Pro Gly Ala Pro Gly Ala Pro Gly Ala Pro Gly Pro  
 50 55 60

Val Gly Pro Ala Gly Lys Ser Gly Asp Arg Gly Glu Thr Gly Pro Ala  
 65 70 75 80

Gly Pro Ala Gly Pro Val Gly Pro Val Gly Ala Arg Gly Pro Ala Gly  
 85 90 95

Pro Gln Gly Pro Arg Gly Asp Lys Gly Glu Thr Gly Glu Gln Gly Asp  
 100 105 110

Arg Gly Ile Lys Gly His Arg Gly Phe Ser Gly Leu Gln Gly Pro Pro  
 115 120 125

Gly Pro Pro Gly Ser Pro Gly Glu Gln Gly Pro Ser Gly Ala Ser Gly  
 130 135 140

Pro Ala Gly Pro Arg Gly Pro Pro Gly Ser Ala Gly Ala Pro Gly Lys  
 145 150 155 160

Asp Gly Leu Asn Gly Leu Pro Gly Pro Ile Gly Pro Pro Gly Pro Arg  
 165 170 175

Gly Arg Thr Gly Asp Ala Gly Pro Val Gly Pro Pro Gly Pro Gly  
 180 185 190

Pro Pro Gly Pro Pro Gly Pro Pro  
 195 200

<210> 29  
 <211> 100  
 <212> PRT  
 <213> human

<400> 29

Arg Gly Asp Lys Gly Glu Thr Gly Glu Gln Gly Asp Arg Gly Ile Lys  
 1 5 10 15

Gly His Arg Gly 20 a Ser Gly Leu Gln Gly Pro Pro Gly Pro Pro Gly  
 Ser Pro Gly Glu Gln Gly Pro Ser Gly Ala Ser Gly Pro Ala Gly Pro  
 Arg Gly Pro Pro Gly Ser Ala Gly Ala Pro Gly Lys Asp Gly Leu Asn  
 Gly Leu Pro Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Thr Gly  
 Asp Ala Gly Pro Val Gly Pro Pro Gly Pro Gly Pro Pro Gly Pro  
 Pro Gly Pro Pro

<210> 30  
 <211> 62  
 <212> PRT  
 <213> human

<400> 30  
 Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly Pro Ala Gly Glu  
 Arg Gly Glu Gln Gly Pro Ala Gly Ser Pro Gly Phe Gln Gly Leu Pro  
 Gly Pro Ala Gly Pro Pro Gly Glu Ala Gly Lys Pro Gly Glu Gln Gly  
 Val Pro Gly Asp Leu Gly Ala Pro Gly Pro Ser Gly Ala Arg

<210> 31  
 <211> 251  
 <212> PRT  
 <213> human

<400> 31  
 Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly Pro Ala Gly Glu  
 Arg Gly Glu Gln Gly Pro Ala Gly Ser Pro Gly Phe Gln Gly Leu Pro  
 Gly Pro Ala Gly Pro Pro Gly Glu Ala Gly Lys Pro Gly Glu Gln Gly  
 Val Pro Gly Asp Leu Gly Ala Pro Gly Pro Ser Gly Ala Arg Gly Glu  
 Arg Gly Phe Pro Gly Glu Arg Gly Val Gln Gly Pro Pro Gly Pro Ala

Gly Pro Arg Gly a Asn Gly Ala Pro Gly Asn Asp Gly Ala Lys Gly  
 5 90 95  
 Asp Ala Gly Ala Pro Gly Ala Pro Gly Ser Gln Gly Ala Pro Gly Leu  
 100 105 110  
 Gln Gly Met Pro Gly Glu Arg Gly Ala Ala Gly Leu Pro Gly Pro Lys  
 115 120 125  
 Gly Asp Arg Gly Asp Ala Gly Pro Lys Gly Ala Asp Gly Ser Pro Gly  
 130 135 140  
 Lys Asp Gly Val Arg Gly Leu Thr Gly Pro Ile Gly Pro Pro Gly Pro  
 145 150 155 160  
 Ala Gly Ala Pro Gly Asp Lys Gly Glu Ser Gly Pro Ser Gly Pro Ala  
 165 170 175  
 Gly Pro Thr Gly Ala Arg Gly Ala Pro Gly Asp Arg Gly Glu Pro Gly  
 180 185 190  
 Pro Pro Gly Pro Ala Gly Phe Ala Gly Pro Pro Gly Ala Asp Gly Gln  
 195 200 205  
 Pro Gly Ala Lys Gly Glu Pro Gly Asp Ala Gly Ala Lys Gly Asp Ala  
 210 215 220  
 Gly Pro Pro Gly Pro Ala Gly Pro Ala Gly Pro Pro Gly Pro Ile Gly  
 225 230 235 240  
 Asn Val Gly Ala Pro Gly Ala Lys Gly Ala Arg  
 245 250

<210> 32  
 <211> 43  
 <212> DNA  
 <213> human

<400> 32  
 agcttctaga ttattagggg ggaccagggg gaccaggagg tcc

43

<210> 33  
 <211> 662  
 <212> PRT  
 <213> human

<400> 33  
 Glu Ala Gly Leu Pro Gly Ala Lys Gly Leu Thr Gly Ser Pro Gly Ser  
 1 5 10 15  
 Pro Gly Pro Asp Gly Lys Thr Gly Pro Pro Gly Pro Ala Gly Gln Asp  
 20 25 30  
 Gly Arg Pro Gly Pro Pro Gly Pro Pro Gly Ala Arg Gly Gln Ala Gly  
 35 40 45

Val Met Gly Phe 50 Gly Pro Lys Gly Ala Ala Gly Glu Pro Gly Lys 60  
 Ala Gly Glu Arg Gly Val Pro Gly Pro Pro Gly Ala Val Gly Pro Ala 65 70 75 80  
 Gly Lys Asp Gly Glu Ala Gly Ala Gln Gly Pro Pro Gly Pro Ala Gly 85 90 95  
 Pro Ala Gly Glu Arg Gly Glu Gln Gly Pro Ala Gly Ser Pro Gly Phe 100 105 110  
 Gln Gly Leu Pro Gly Pro Ala Gly Pro Pro Gly Glu Ala Gly Lys Pro 115 120 125  
 Gly Glu Gln Gly Val Pro Gly Asp Leu Gly Ala Pro Gly Pro Ser Gly 130 135 140  
 Ala Arg Gly Glu Arg Gly Phe Pro Gly Glu Arg Gly Val Gln Gly Pro 145 150 155 160  
 Pro Gly Pro Ala Gly Pro Arg Gly Ala Asn Gly Ala Pro Gly Asn Asp 165 170 175  
 Gly Ala Lys Gly Asp Ala Gly Ala Pro Gly Ala Pro Gly Ser Gln Gly 180 185 190  
 Ala Pro Gly Leu Gln Gly Met Pro Gly Glu Arg Gly Ala Ala Gly Leu 195 200 205  
 Pro Gly Pro Lys Gly Asp Arg Gly Asp Ala Gly Pro Lys Gly Ala Asp 210 215 220  
 Gly Ser Pro Gly Lys Asp Gly Val Arg Gly Leu Thr Gly Pro Ile Gly 225 230 235 240  
 Pro Pro Gly Pro Ala Gly Ala Pro Gly Asp Lys Gly Glu Ser Gly Pro 245 250 255  
 Ser Gly Pro Ala Gly Pro Thr Gly Ala Arg Gly Ala Pro Gly Asp Arg 260 265 270  
 Gly Glu Pro Gly Pro Pro Gly Pro Ala Gly Phe Ala Gly Pro Pro Gly 275 280 285  
 Ala Asp Gly Gln Pro Gly Ala Lys Gly Glu Pro Gly Asp Ala Gly Ala 290 295 300  
 Lys Gly Asp Ala Gly Pro Pro Gly Pro Ala Gly Pro Ala Gly Pro Pro 305 310 315 320  
 Gly Pro Ile Gly Asn Val Gly Ala Pro Gly Ala Lys Gly Ala Arg Gly 325 330 335  
 Ser Ala Gly Pro Pro Gly Ala Thr Gly Phe Pro Gly Ala Ala Gly Arg 340 345 350  
 Val Gly Pro Pro Gly Pro Ser Gly Asn Ala Gly Pro Pro Gly Pro Pro 355 360 365

Gly Pro Ala Gly : s Glu Gly Gly Lys Gly Pro Arg Gly Glu Thr Gly  
 370 375 380  
 Pro Ala Gly Arg Pro Gly Glu Val Gly Pro Pro Gly Pro Pro Gly Pro  
 385 390 395 400  
 Ala Gly Glu Lys Gly Ser Pro Gly Ala Asp Gly Pro Ala Gly Ala Pro  
 405 410 415  
 Gly Thr Pro Gly Pro Gln Gly Ile Ala Gly Gln Arg Gly Val Val Gly  
 420 425 430  
 Leu Pro Gly Gln Arg Gly Glu Arg Gly Phe Pro Gly Leu Pro Gly Pro  
 435 440 445  
 Ser Gly Glu Pro Gly Lys Gln Gly Pro Ser Gly Ala Ser Gly Glu Arg  
 450 455 460  
 Gly Pro Pro Gly Pro Met Gly Pro Pro Gly Leu Ala Gly Pro Pro Gly  
 465 470 475 480  
 Glu Ser Gly Arg Glu Gly Ala Pro Ala Ala Glu Gly Ser Pro Gly Arg  
 485 490 495  
 Asp Gly Ser Pro Gly Ala Lys Gly Asp Arg Gly Glu Thr Gly Pro Ala  
 500 505 510  
 Gly Pro Pro Gly Ala Pro Gly Ala Pro Gly Ala Pro Gly Pro Val Gly  
 515 520 525  
 Pro Ala Gly Lys Ser Gly Asp Arg Gly Glu Thr Gly Pro Ala Gly Pro  
 530 535 540  
 Ala Gly Pro Val Gly Pro Val Gly Ala Arg Gly Pro Ala Gly Pro Gln  
 545 550 555 560  
 Gly Pro Arg Gly Asp Lys Gly Glu Thr Gly Glu Gln Gly Asp Arg Gly  
 565 570 575  
 Ile Lys Gly His Arg Gly Phe Ser Gly Leu Gln Gly Pro Pro Gly Pro  
 580 585 590  
 Pro Gly Ser Pro Gly Glu Gln Gly Pro Ser Gly Ala Ser Gly Pro Ala  
 595 600 605  
 Gly Pro Arg Gly Pro Pro Gly Ser Ala Gly Ala Pro Gly Lys Asp Gly  
 610 615 620  
 Leu Asn Gly Leu Pro Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg  
 625 630 635 640  
 Thr Gly Asp Ala Gly Pro Val Gly Pro Pro Gly Pro Pro Gly Pro Pro  
 645 650 655  
 Gly Pro Pro Gly Pro Pro  
 660